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THE UNIVERSITY OF ALBERTA

Strategies for the Immobilization of Chloroperoxidase

by

Tenshuk Ange Kadima

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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(Supervisor)

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Date: 8 September 1989

DEDICATION.

I dedicate this thesis to my wonderful wife, Mavis, and our precious young ones, Elenga and Daniel, for their Love, Patience, and Understanding.

ABSTRACT.

Chloroperoxidase (CPO), an extracellular heme glycoprotein, was purified from *Caldariomyces fumago* CMI 89362 and covalently bound to derivatized 200-400-mesh controlled-pore glass (170 Å pore size). In a modification of established methodology, acid-washed glass was aminated using aminopropyltriethoxysilane, and the enzyme was ionically bound to this aminopropyl-glass at low ionic strength. Further treatment with glutaraldehyde covalently crosslinked the enzyme on the glass beads.

A colorimetric enzyme assay, based on the peroxidation of tetramethyl-*p*-phenylenediamine (TMPD) by CPO, was developed to quantitate the activity of the immobilized enzyme. No elution of bound activity from glass beads could be detected with a variety of washings. The levels of enzyme loaded on glass beads were higher than those reported for related systems, and the specific activity of the immobilized enzyme was highest at low enzyme to carrier ratio.

The characterization of immobilized and soluble CPO indicated that the temperature stability and pH activity profiles of both were similar. The pH activity profiles showed two peaks, at pH 2.8 and 6.0, corresponding respectively to the halide- dependent and halide-independent demethylation activities of CPO. Both enzymes displayed greater stability in the pH range of 3.0 to 6.0 during a 1-month storage at 5°C. The storage of the enzyme in the presence of a substrate, phenol, protected the enzyme from alkaline denaturation at pH 7.0. The operational stability of immobilized and soluble enzymes was maximal at pH values near neutral, on the acidic side; at low concentration of hydrogen peroxide (1 mM), loss of enzyme activity occurred at acidic pH values. The kinetic studies indicated that CPO conformed to the postulated "peroxidase ping-pong" kinetic mechanism, and no Michaelis-Menten constants could be determined for H_2O_2 or TMPD.

Unlike the soluble enzyme, immobilized CPO used in a shaking-batch reactor was an inefficient biocatalyst in the removal of phenol from aqueous solutions - because the reaction products complexed with the carrier-bound enzyme. However, promising results were obtained from a preliminary study on phenol oxidation by immobilized CPO in a packed-bed reactor. More studies are required on this system, in which the contact time between the products and the enzyme can be reduced.

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I would like to offer my heartfelt gratitude to my supervisor Dr. M.A. Pickard for all his help and encouragement during the course of this project. His keen insights and timely advice were crucial to the successful completion of my thesis. I would also like to thank all the members of my committee, whose constructive criticism on my work, and helpful advice on personal and academic matters, were of great importance for the completion of this thesis.

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LIST OF ABBREVIATIONS.

Abbreviation	DESCRIPTION
A-CPG	aminopropyl-controlled-pore glass
APES	3-aminopropyltriethoxysilane
CPG	controlled-pore glass. The pore size may also appear as in CPG-75 for a 75 Å pore size porous glass
CPO	chloroperoxidase
HRPO	horseradish peroxidase
GC	gas chromatography
P-450	cytochrome P-450 monooxygenase
TMPD	N,N,N',N'-tetramethyl-*p*-phenylenediamine

1. LITERATURE REVIEW AND OBJECTIVES.

1.1 Introduction.

Peroxidases are classified as the group of heterogenous enzymes that catalyze the hydroperoxide-dependent oxidation of a wide spectrum of organic and inorganic compounds. They are versatile biocatalysts. Some peroxidases can, in addition to the oxidation of their typical substrates, carry out a variety of reactions, such as the halogenation of organic compounds, disproportionation of hydrogen peroxide, and demethylation of aromatic amines. The latter two reactions were first believed to be typical of catalase and cytochrome P-450 monooxygenase (P-450) , respectively. Like most peroxidases, both catalase and P-450 are heme-containing glycoproteins. Because of their shared similarities with peroxidases, these two enzymes have often appeared in peroxidase literature, where comparative studies to elucidate the function-structure relationships of heme-containing peroxidases have been reported. Citations to most of the above statements will be found later in this chapter at appropriate places.

Studies of peroxidases have a long history. Horseradish peroxidase (HRPO) in particular has played a historically important role in the development of the modern concept of the nature of an enzyme and the role of metal ions as documented by a famed controversy in the 1920's and 1930's (Dunford and Stillman 1976). Several thousands of research papers have been written on peroxidases. Reviewing this literature without making recourse to many excellent earlier reviews and focussing on specific areas can be an enormous challenge to an author. Therefore, this literature review will attempt to highlight some of the history of peroxidases before 1960. Original references of historical interest may be found in reviews written by Dunford and Stillman (1976), Saunders (1973), and Saunders *et al.* (1964). The purpose of this chapter is also to point out the wide distribution of peroxidases in biological systems and to give an

account of selected topics that are directly related to this thesis project. These include the structure, properties, mechanisms of action, and some applications of peroxidases, with some emphasis on chloroperoxidase (CPO) studies; and an overview of immobilized enzyme technology. To conclude, the objectives and rationale of this M.Sc. thesis project will be presented.

1.2 Peroxidases.

1.2.1 Historical review.

The first indication of occurrence of the peroxidatic activity in biological systems dates back to the nineteenth century. In 1810, Planche observed that guaiacum turned to an intense blue color in the presence of fresh root of horseradish. This bluing reaction was previously noted in people's mouths in 1809, probably due to the use of guaiacum as a dentifrice. This observation coincides with the first report of a peroxidase reaction. In 1855, Schönbein recorded the hydrogen peroxide-dependent oxidation of organic compounds by "substances" extracted from plants and animals. It was not until the end of the nineteenth century that the name "peroxidase" was first given to this "substance", after its isolation from pus by Linossier in 1898.

Although peroxidases were known before 1900, not much progress in understanding the properties of the enzymatic intermediates and the kinetics of peroxidatic activity was made until the 1940's and 1950's, with the pioneering work of B. Chance, P. George, and other talented researchers. In 1931, Hand and co-workers showed that HRPO was a hemoprotein. Later in 1942, Theorell and co-workers showed that the HRPO heme prosthetic group consisted of ferric protoporphyrin IX. The studies on the reaction of HRPO with hydrogen peroxide by Keilin and Mann in 1937, and Theorell in 1941, resulted in the discovery of the different enzymic species

of this classical peroxidase, which were known as compound II and compound I, respectively. This discovery was of great importance in the history of biochemistry since these components were the first observable enzyme-substrate complexes. In early 1950's, Chance and George further investigated the spectra of the two species of HRPO in the 400 nm (Soret) region, and the kinetics of some HRPO reactions.

In the past two decades, intensive efforts have been directed towards an understanding of the reaction mechanisms of peroxidases (Dunford *et al.* 1987, Griffin and Ting 1978, Jenzer *et al.* 1986, Kedderis *et al.* 1986, Metodiewa *et al.* 1989, Neidleman and Geigert 1986, Yamazaki 1974). Progress has also been made in deciphering the active site structures of peroxidases, and understanding the interrelationship of metal structure and protein environment in determining the catalytic activity of some of these enzymes (Dawson 1988, Dawson and Sono 1987, Ortiz de Montellano 1987, Ortiz de Montellano *et al.* 1987).

1.2.2 Sources of peroxidases.

Peroxidases are ubiquitous in biological systems. A plausible hypothesis accounting for their wide occurrence is that they were first used by primordial organisms in their defense mechanisms against oxidation by toxic oxygen compounds (Klebanoff and Clark 1978). Their more specialized functions, including attack on invading foreign organisms, as in the case of myeloperoxidase, evolved later once survival from oxidation was firmly established.

The extensive list provided by Saunders *et al.* (1964) indicated the impressive distribution of these enzymes in plants and animals. Horseradish roots and the sap of fig trees are the richest source of plant peroxidases, such as HRPO. Vertebrate tissues and body fluids have been the major source of animal peroxidases, such as thyroid peroxidase, eosinophilic peroxidase, lactoperoxidase, and myeloperoxidase.

Peroxidases have also been isolated from fungi, algae, and bacteria. CPO from *Caldariomyces fumago* (Shaw and Hager 1959), cytochrome c peroxidase from aerobically grown baker's yeast (Saunders *et al.* 1964), and lignin peroxidase from *Phanaerochete chrysosporium* (Glenn *et al.* 1983, Tien and Kirk 1983), are some of the most studied fungal peroxidases. Enzymes with similar activity have also been isolated from bacteria (Weisner *et al.* 1986).

1.2.3 Properties of peroxidases.

The properties of peroxidases have been reviewed by Dunford (1982, 1989), Dunford and Stillman (1976), Morrison and Schonbaum (1976), Neidleman and Geigert (1986), and Saunders *et al.* (1964). Due to the heterogeneity of this group of enzymes, it is difficult to describe definitively the basic characteristics of peroxidases. However, because most of the studies on peroxidases have been carried out with HRPO, the generalization of HRPO properties has often served as the core of the basic characteristics of these enzymes.

Generally, peroxidases are hemoproteins with hemin or ferriprotoporphyrin IX as the prosthetic group. Exceptions are glutathione peroxidase and myeloperoxidase which lack the ferriprotoporphyrin IX prosthetic group (Neidleman and Geigert 1986). Glutathione peroxidase contains one atom of selenium per peptide (Ladenstein *et al.* 1979), and myeloperoxidase contains two hemes per molecule (Schultz 1980). Heme (Sievers 1979) and non-heme (Dumontet and Rousset 1983) lactoperoxidases have been discovered as well. The number of known non-heme peroxidases is however growing (Liu *et al.* 1987, Neidleman and Geigert 1986, Weisner *et al.* 1988).

The heme ligands of most peroxidases have been identified, except for the nature of the sixth ligand which still remains a topic of controversy. In most peroxidases, four of the six heme ligands are provided by the porphyrin, and a histidine

is at the fifth coordination position (Dunford 1982). However, a thiolate has been identified at the fifth heme coordination position in cytochrome c peroxidase (Poulos *et al.* 1978) and CPO (Dawson and Sono 1987). Based on evidence accumulated over the past decade and a half, the sixth coordination position of the iron(III) of the native peroxidase is unoccupied, unlike that of the native metmyoglobin which is occupied by water (Dunford 1989). However, Sono *et al.* (1986) did a comparative study of the magnetic circular dichroism (MCD) spectra of the high-pH derivative of ferric CPO and those of the imidazole adducts of ferric P-450 and low-pH CPO. They found spectroscopic evidence indicating that the sixth ligand to the high-pH ferric CPO was a histidine.

Comparisons of amino-acid sequence near the active site of different peroxidases have indicated some homology in this region between turnip peroxidase, cytochrome c peroxidase, HRPO and lignin peroxidase (Tien and Tu 1987). These peroxidases contain two histidine residues and an arginine which are thought to be essential for their activity (Dunford and Stillman 1976, Poulos *et al.* 1986). One of the histidines (proximal) is the axial ligand of the heme, and the other histidine (distal) (along with the arginine) is proposed to be involved in charge stabilization during reaction of the heme with H_2O_2 (Poulos and Kraut 1980).

Most peroxidases are glycoproteins, with molecular weights ranging from 12 K (NADH peroxidase) to 149 K (myeloperoxidase) (Dunford and Stillman 1976). Cytochrome c peroxidase and myeloperoxidase do not have a carbohydrate moiety. HRPO (Aibara *et al.* 1982), CPO (Morris and Hager 1966), and lignin peroxidase (Glenn *et al.* 1983, Tien and Kirk 1983) all are monomeric glycoproteins with molecular weigths of about 42 K, corresponding to the average size of classical peroxidases. Several peroxidases contain multiple subunits. Bromoperoxidases from *Pseudomonas aureofaciens* (van Pee and Lingens 1985) and *Pseudomonas capitatus*

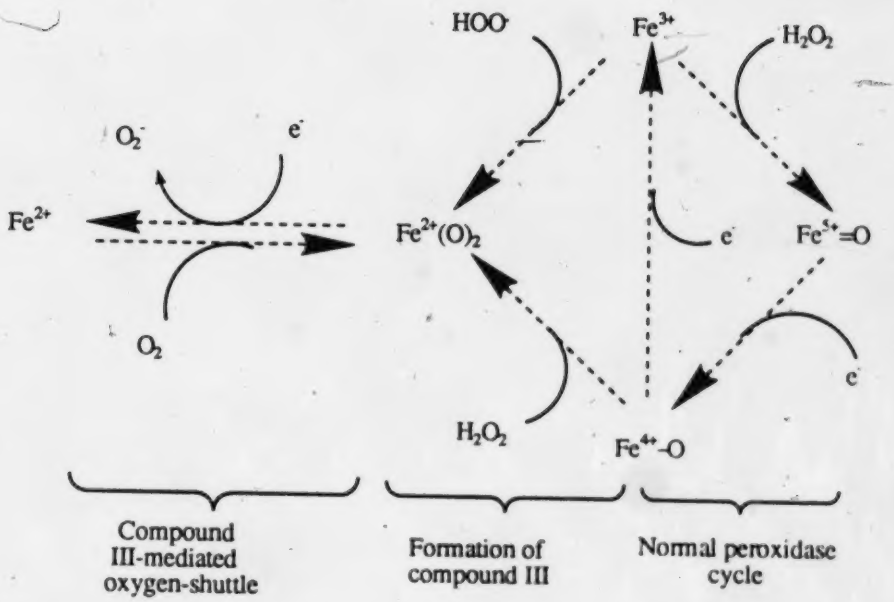
(Manthey and Hager 1981) are two such enzymes.

Peroxidases show little specificity towards the substrates they oxidize. Unlike other enzymes, peroxidases are not specific to a certain functional group or even to a small family of compounds (Saunders *et al.* 1964). They can oxidize substrates as simple as potassium iodide or potassium ferrocyanide and as complex as veratryl alcohol (Palmer *et al.* 1987). Most peroxidases can catalyze the oxidation of one or more of the following halide ions: I^-, Br^-, and Cl^-, but not F^- (Neidleman and Geigert 1986). A known exception is glutathione peroxidase which is a non-halogenating peroxidase. Lignin peroxidase can oxidize not only all the substrates oxidized by HRPO, but also methoxylate aromatic compounds that are not substrates for HRPO (Palmer *et al.* 1987). Cytochrome c peroxidase differs from other peroxidases in that it is especially active in catalyzing the one-electron oxidation of a protein rather than a small molecule (Ortiz de Montellano 1987). However, this enzyme can also catalyze the oxidation of some typical HRPO substrates by hydrogen peroxide (Saunders *et al.* 1964).

The catalytic intermediates of a classical peroxidase have been identified and characterized, and the interrelations between the five redox states (Figure 1.1) have been determined (Yamazaki 1974). In the normal catalytic cycle, the ferric form of the peroxidase is oxidized by hydrogen peroxide to a two-electron deficient oxy-ferryl state known as compound I, which is the active oxidant in peroxidase reactions. One of the two oxidation equivalents is invariably associated with the Fe^{3+} to Fe^{4+} redox change. The second oxidation equivalent is, however, placed on the porphyrin in some peroxidases, such as horseradish peroxidase to form a radical cation (Dolphin *et al.* 1971, Thanabal *et al.* 1988), or on the protein in others, such as cytochrome c peroxidase to form a protein radical (Yonetani and Ray 1965). Compound I is then reduced by two consecutive single-electron transfer steps to the ground state via

Figure 1.1. Interrelations between the redox states of peroxidase (Reproduced from Palmer *et al.* 1987).

In the normal catalytic cycle, the ferric form of the enzyme is oxidized by hydrogen peroxide to a two-electron deficient oxy-ferryl state ($Fe^{5+}=O$, or compound I). Compound I is then reduced by two consecutive single-electron transfer steps to a ground state via compound II (Fe^{4+}-O). Under appropriate conditions, peroxidase is converted into a less catalytically active form (compound III) by excessive amount of hydrogen peroxide. Compound III can set up a shuttle with the ferrous form that results in the oxidation of substrates and reduction of dioxygen to superoxide, or can be converted back to the ferric ground state.



Reproduced from Scheme 1
Palmer et al. 1987.

compound II. The reaction of compound II with the hydrogen donor is the rate-limiting step (Chance 1943, Dunford 1989). In the absence of the hydrogen donor, compound I converts slowly to compound II. On the other hand, in the presence of excessive amount of hydrogen peroxide, the enzyme is converted into a less catalytically active form known as compound III (Palmer *et al.* 1987). This state can also be achieved by reacting the ferric form of the enzyme with perhydroxyl radicals. The role of compound III in the catalytic cycle is complex. Compound III can set up a shuttle with the ferrous form that results in the oxidation of the substrates and the reduction of dioxygen to superoxide, or can be converted back to the ferric ground state (Manthey and Hager 1985, Yamazaki and Yokota 1973). Similarly, the peroxidatic cycle and intermediate compounds I, II, and III, involved in the catalytic mechanism of CPO have also been described (Lambeir and Dunford 1985, Nakajima *et al.* 1985, Palcic *et al.* 1980, Sono *et al.* 1985). And recently, the direct formation of compound II in the reaction with superoxide has been reported for CPO (Metodiewa *et al.* 1989).

1.2.4 Kinetics of the peroxidatic reactions.

Steady state kinetic experiments have been used to deduce the kinetic mechanisms of peroxidase reactions (Saunders *et al.* 1964), and determine the rate constants for rate-controlling steps (Dunford and Stillman 1976). Peroxidases carry out two-substrate reactions, and as discussed above, both compounds I and II are reduced by the same substrate as shown in the enzymatic cycle below (from Dunford 1989):

$$HRPO + H_2O_2 \xrightarrow{k_1} HRPO\text{-}I + H_2O \qquad (1)$$

$$HRPO\text{-}I + AH_2 \xrightarrow{k_2} HRPO\text{-}II + {}^{\bullet}AH \qquad (2)$$

$$\text{HRPO-II} + AH_2 \xrightarrow{k_3} \text{HRPO} + {}^{\bullet}AH + H_2O \quad (3)$$

where HRPO-I and HRPO-II are compounds I and II, AH_2 is a reducing substrate and $^{\bullet}AH$ is a free radical product. The kinetics of peroxidase reaction are often obscured since both reductive steps of compound I to regenerate the native enzyme tend to occur spontaneously even in the absence of the hydrogen donor (Saunders *et al.* 1964). The classical ping-pong scheme is a reversible, ordered, two-substrate:two product mechanism (Segel 1975). Unlike this classical (conventional) ping-pong mechanism, the peroxidase ping-pong scheme shown above contains no hint of reversibility in the equations (1-3); compound I is usually reduced the faster of the two; and the conversion of compound II to the native enzyme is often the rate-limiting step (Dunford 1989). The steady-state equation corresponding to equations (1-3) is therefore a modified form of ping-pong kinetics (Dunford 1989, Dunford and Stillman 1976). Theoretical considerations of this modified ping-pong kinetics indicate that, since k_2 is about 10 times greater than k_3 for most substrates of HRPO, no Michaelis-Menten kinetic constants can be derived for HRPO and peroxidases in general (Dunford 1989). However, experimental data have been reported for a number of peroxidase-catalyzed reactions, where Km values for both the oxidizing and reducing substrates were derived (de Boer and Wever 1988, Dunford and Stillman 1976, Kedderis and Hollenberg 1983). The rate constants of each individual steps have also been determined with stopped-flow experiments (Dunford 1989, Lambeir and Dunford 1983a, Saunders *et al.* 1964).

1.2.5 Chloroperoxidase.

CPO is a 42 K heme-containing glycoprotein secreted by *Caldariomyces*

fumago (Morris and Hager 1966, Shaw and Hager 1959). Upon fructose induction, the enzyme is secreted at levels up to 500 units/liter into the culture filtrate (Pickard 1981) - being one of the highest protein secretion levels known - and at a purity level of about 85%. No enzyme is produced when glucose is used to provide carbon to cells, and the CPO mRNA pool varies in concert with the levels of secreted enzyme activity (Axley *et al.* 1986). The structural gene of CPO has been isolated, cloned and sequenced by Hager and co-workers (Fang *et al.* 1986, Nuell *et al.* 1988). The peptide sequence indicated that CPO is a 321 amino acid long protein, with predominant acidic residues.

CPO is a versatile catalyst (Dawson 1988). It can function as a peroxidase by catalyzing the oxidation of substrates with simultaneous reduction of hydrogen peroxide to water. It can also function as a catalase by disproportionating H_2O_2 to O_2 and H_2O (Thomas *et al.* 1970). In addition to the usual peroxidase and catalase activities exhibited by other peroxidases (Frew and Jones 1984), CPO has a unique halogenase activity. It catalyzes the hydrogen peroxide-dependent oxidation of chloride ion, in addition to bromide and iodide ions, resulting in the formation of carbon-halogen bonds with halogen acceptors such as ß-diketones (Hager *et al.* 1966). Most peroxidases are only able to brominate and iodinate organic compounds by using halide ions as a source of the halogen (Dawson and Sono 1987, Neidleman and Geigert 1986), eosinophilic peroxidase is an exception (Bardsley 1985). CPO can also carry out the hydrogen peroxide-independent chlorination of substrates using chlorite as the halogen source (Hollenberg *et al.* 1974).

CPO has also been found to catalyze the N-dealkylation of arylamines (Griffin and Ting 1978; Kedderis *et al.* 1980, 1986), the epoxidation of alkenes, and the conversion of sulfides to sulfoxides, reactions that are typical of the P-450 (McCarthy

and White 1983, Weiner 1986). During the epoxidation of styrene, Ortiz de Montellano *et al.* (1987) showed that the mechanism of oxo transfer to styrene involved a direct interaction of the substrate with the iron-bound oxo atom, and that the oxygen atom transferred derived from H_2O_2. Therefore they concluded that, in addition to the one-electron reactions catalyzed by CPO as a peroxidase, CPO can carry out two-electron reactions as a peroxygenase.

The coordination structure of the heme iron of CPO has been a subject of extensive research over the past 15 years. As reviewed recently (Dawson and Sono 1987), a consensus has been reached that CPO, like P-450, has a thiolate axial ligand. This conclusion was based on the results obtained from the spectral comparisons of heme proteins and models with various CPO states (Bangcharoenpaurpong *et al.* 1986, Kau *et al.* 1986), and from the knowledge of the primary sequence of CPO, directly determined from the CPO gene (Fang *et al.* 1986).

Despite the close similarities in active site structures of P-450 and CPO, these two enzymes support different chemistry. This observation suggests that additional factors other than just the metal coordination structure play important roles in determining the catalytic differences (Dawson 1988). Studies by Sono *et al.* (1986) and Ortiz de Montellano *et al.* (1987) have revealed differences in the heme environment of CPO relative to P-450 that may correlate with their enzymatic activities. Unlike P-450, CPO has a more polar peroxidase-type heme environment surrounding a P-450-type metal coordination structure.

1.2.6 Applications of peroxidases.

Enzymes in general participate in many chemical reactions that occur in living systems. They have been exploited by human beings since ancient times, well before their nature and function, or even the animals, plants, and microorganisms from which

they were derived were known and understood (Kennedy and Cabral 1983). It was not until the twentieth century that these proteins or glycoproteins were shown to be involved in all fermentation processes. As more is known about the nature and chemical properties of enzymes, the use of these biocatalysts has gradually been extended in a variety of fields, such as brewing, food production, textiles, pharmaceuticals, and medicine (Kennedy and Cabral 1983), and removal of toxic wastes (Neidleman and Geigert 1986).

Peroxidases, because of their wide substrate specificity and multiple catalytic activities, have a wide range of potential commercial applications. Martyn *et al.* (1981) evaluated the commercial potential for haloperoxidases, and predicted that the use of enzymatic halogenations presented almost unlimited possibilities for the production of useful chemicals in the future. As halogenases, these enzymes can be used as a synthetic tool in the production of industrial chemicals (Franssen and van der Plas 1984). They could also find use in the areas of analytical diagnostics (Grime and Lockhart 1979, Manthey *et al.* 1984, Neidleman and Geigert 1986), and production of *in situ* antibacterial agents (Bjorck 1982, Hasegawa and Kobayashi 1983).

The use of these enzymes as peroxidases has been well established in synthetic chemistry. The syntheses of coumarinolignans by oxidative coupling with HRPO (Lin and Cordell 1984), and of antihypertensive glycoside derivatives by oxidative dimerization of coniferyl alcohol with CPO (Sih 1978), are some examples. It was also postulated that peroxidases could find greater commercial application in the area of pollution control, where they have been shown to remove carcinogenic aromatic amines from industrial aqueous effluents (Klibanov and Morris 1981), phenols from coal-conversion waste waters (Carmichael *et al.* 1985, Klibanov *et al.* 1983), low molecular weight color bodies from pulp mill effluents (Paice and Jurasek 1984), and flavored aromatics from drinking waters (Fiessinger *et al.* 1984).

1.3 Enzyme immobilization.

The use of enzymes in industrial applications has been generally limited because of their relative instability, high isolation and purification costs, and the difficulty in recovering active enzymes from the reaction mixture after completion of the catalytic process (Kennedy and Cabral 1987). Peroxidases are no exception. Five years later, contrary to the glowing evaluation by Martyn *et al.* (1981), Neidleman and Geigert (1986) found that peroxidases had still very few commercial applications. In their analysis, Neidleman and Geigert (1986) identified a number of technological and operational problems which required satisfactory solutions. The two major problems inherent to peroxidase processes were: (i) obtaining adequate supplies of the desired biocatalyst at the required degree of purity, and (ii) optimizing operational conditions to yield economically useful activity and lifetime. The latter has been the focus of this thesis.

Larger quantities of enzymes are needed for commercial applications. The production of plant enzymes, such as HRPO, has generally been less cost-effective than microbial enzymes (Chibata and Tosa 1981, Klibanov *et al.* 1983), even though alternative technologies such as cell culture and recombinant DNA techniques are now being applied towards the production of large quantities of enzyme (Neidleman and Geigert 1986). The HRPO system is of interest, because it is the most studied peroxidase system (Dunford 1989). Also, some economic aspects of its use in industrial processes have been evaluated (Alberti and Klibanov 1981). However, Pickard and co-workers (Carmichael 1987, Carmichael and Pickard 1989, Pickard 1981) have shown that the fungal enzyme, CPO, could be produced at much higher levels than horseradish peroxidase, and at lower costs. Therefore, on a mass to mass

basis, the CPO system might serve as an alternative for the proposed commercial applications of HRPO.

In this section a brief discussion on some of the various methodologies of enzyme immobilization and characteristics of immobilized enzyme systems will be presented. Of necessity, many important aspects of the field of immobilized enzyme technology will not be adequately covered. For complete reviews, the reader is refered to other reviews (Bernath and Venkatasubramanian 1986, Kennedy and Cabral 1983, 1987) or some books that have appeared in the last few years (Chibata *et al.* 1982, Mosbach 1987, Weetall and Royer 1980, Wingard *et al.* 1976).

1.3.1 Historical perspective.

It is difficult to assess the current status of immobilized enzyme technology without an appreciation of its roots (Kennedy and Cabral 1983). This section will attempt to highlight some of the important events in the history of enzyme immobilization. An extensive survey of the field from its rather diffuse and unspectacular beginnings to its present status as a sophisticated technology with the potential for dramatically affecting our future life-styles was reviewed by Goldstein and Katchalski-Katzir (1976). Another historical account of enzyme immobilization technology has been provided in a particularly useful review by Kennedy and Cabral (1983).

Nelson and Griffin were first to report the preparation of an immobilized enzyme in 1916, with the adsorption of invertase on charcoal. In 1930's, immunologists became the first scientists to make use of this phenomenon by using adsorbed antigens for the isolation of specific antibodies. They also carried out initial attempts to covalently fix proteins onto water-insoluble carriers (Kennedy and Cabral 1983). In 1953, Grubhofer and Schleith reported the first attempt to immobilize an

enzyme for the purpose of improving its properties for a particular application, although it was known for a long time that enzymes in water-insoluble form show catalytic activity (Kennedy and Cabral 1983).

Subsequent progress in the field took place in the second half of this century with the development of new immobilization techniques, and contributions of chemical engineers in the design and analysis of continuous heterogeneous catalytic reactors. In the early 1960's, Katchalsky-Katzir and his co-workers at the Weizmann Institute of Science, Israel, and Manecke carried out extensive studies on new immobilization techniques using a number of different carriers (Kennedy and Cabral 1983). From mid-1960's on, attempts were made to redesign carriers to achieve optimal binding and enzyme stability (Kennedy and Cabral 1983); milder and more general immobilization techniques as alternative to covalent methods were developed (Bernfeld and Wan 1963, Chang 1964); and immobilized enzymes in continuous reactors were studied (Lilly *et al.* 1966). By the end of the 1960's, many of the fundamental principles of immobilized enzyme technology had been developed, and emphasis began to shift towards practical applications (Kennedy and Cabral 1983).

Currently, a considerable number of processes using immobilized biocatalysts are now firmly established and several reliable methods are available for binding enzymes to support materials for routine use in the laboratory and industrial processes (Bernath and Venkatasubramanian 1986). Two important commercial applications are the conversion of glucose to fructose by glucose isomerase - about 4.5 million tons produced per year - and the hydrolysis of penicillins by penicillin acylase (Buchholz and Klein 1987). Further potential remains for widespread use of immobilized enzymes in a variety of industrial, environmental, analytical, and medical applications. Current methods for immobilizing enzymes are numerous and these are discussed in the following sections.

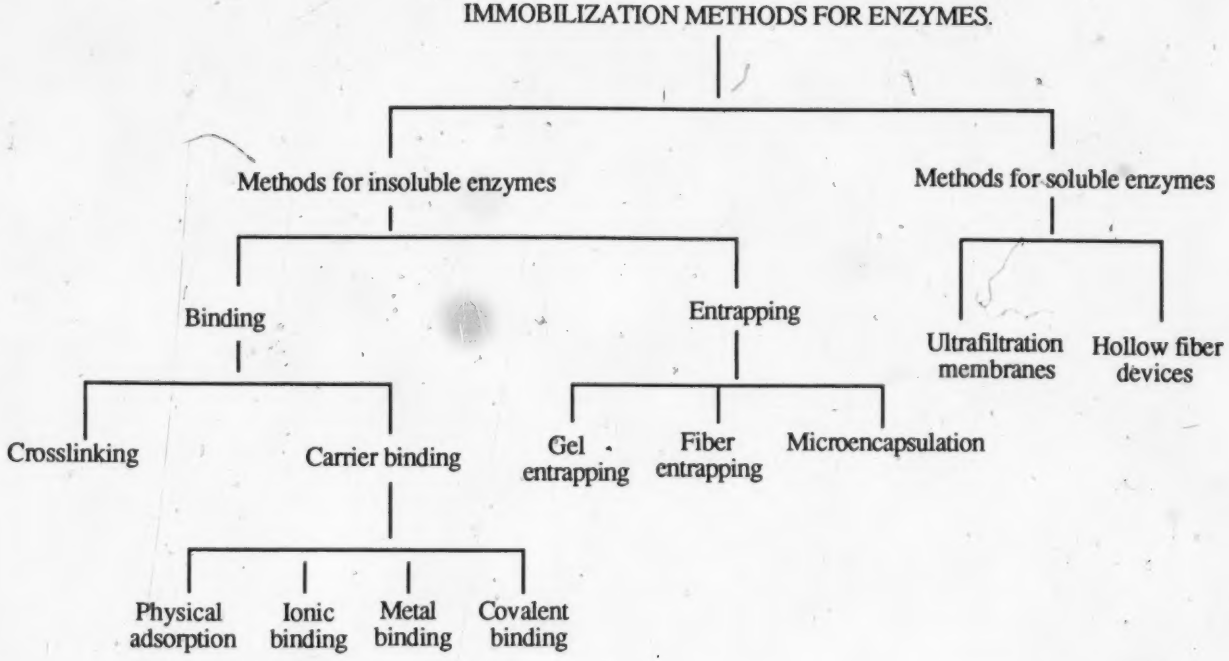
1.3.2 Immobilization methods.

Immobilized enzymes are defined as enzymes which are in the state permitting their continuous or intermittent use over an extended period of time (Poulsen 1983). They are usually classified by the method of binding or localization which allows reuse (Zaborsky 1973).

Immobilized enzymes are also classified on the basis of the nature of the support and the type of coupling reaction. In an attempt to combine the nature of interaction responsible for immobilization and the nature of the support; Kennedy and Cabral (1983) proposed another classification system. This system has been adopted in this thesis, and is not fundamentally different from that recommended by Sundaram and Pye (1972) and Chibata (1978). Figure 1.2 shows this classification system and lists the various individual methods. The two major classes are: (i) methods resulting in the insolubilization of enzyme ("Methods for insoluble enzymes"), and (ii) methods resulting in the physical confinement of soluble enzyme within semipermeable membranes or hollow bore films ("Methods for soluble enzymes"). The "Methods for insoluble enzymes" is divided into the binding and entrapping methods. The binding method is further divided into the crosslinking and carrier binding methods. The carrier binding method, which consists of the binding of enzymes to solid supports, is the oldest (Nelson and Griffin 1916) and the most prevalent method of immobilizing enzymes (Kennedy and Cabral 1983). Based on the binding mode, the carrier binding method can be further divided into four categories: physical adsorption, ionic binding, metal binding, and covalent binding. Only some selected binding methods and some novel immobilization technology are briefly discussed in this review, the reader is refered to the reviews by Kennedy and Cabral (1983, 1987) for a complete detailed discussion on the individual methods.

Figure 1.2. Classification of immobilization methods for enzymes (Reproduced from Kennedy and Cabral 1987).

This classification attempts to combine the nature of interaction responsible for immobilization and the nature of the support. The two major divisions are: (i) "**Methods for insoluble enzymes**" (methods for the preparation of immobilized insoluble enzymes), and (ii) "**Methods for soluble enzymes**" (methods for the preparation of immobilized soluble enzymes).



Reproduced from Figure 7.1
Kennedy and Cabral 1987

1.3.2.1 Adsorption method.

The adsorption method has traditionally refered to the binding of an enzyme by weak attractive forces to an inert carrier which has not been functionalized for ionic or covalent binding (Messing 1975). It is generally regarded as the simplest and most economical procedure for binding enzyme to a solid support (Bernath and Venkatasubramanian 1986). The most important advantage of this binding method from commercial viewpoint is the capability of regenerating the immobilized enzyme activity *in situ* . A variety of inorganic supports have been used to adsorb enzymes, including clay, alumina, sand, glass, and silica. Elution of the enzyme from the carrier may occur, but this can often be reduced by altering the pH, temperature, or ionic strength (Wingard *et al.* 1976).

1.3.2.2 Ionic binding method.

The ionic binding immobilization method is based mainly on ionic binding of enzyme protein to solid supports containing ion-exchange residues (Kennedy and Cabral 1987). This binding mode results in the formation of stronger linkage of the enzyme to the carrier than that obtained by physical adsorption alone. Owing to the ionic character of the linkage between the enzyme and the support and the mild conditions for immobilization, little conformational changes occur with the enzyme, leading to immobilized enzyme with high coupling yields as is the case with physical adsorption. Nevertheless, leakage of enzyme from the carrier may occur in some situations, as with physical adsorption.

1.3.2.3 Covalent binding method.

The covalent binding method is based on the covalent attachment of enzyme to water insoluble carriers. This method has received a great deal of study over the years.

Consequently, there is a considerable amount of information in the literature detailing procedures for a wide variety of matrices and reactive groups (Bernath and Venkatasubramanian 1986; Chibata *et al.* 1982; Kennedy and Cabral 1983, 1987; Messing 1975; Mosbach 1976; Pye and Weetall 1978; Weetall and Royer 1980; Wingard *et al.* 1976). Two key advantages of covalent binding are (i) the stability of the enzyme-carrier bond, which prevent elution of protein into the product stream and (ii) the wide range of choices one has in selecting carrier materials and binding methods. The latter allows a great deal of flexibility in designing an immobilized enzyme system with specific physical and chemical properties (Bernath and Venkatasubramanian 1986). Some of the disadvantages of this method are the relative expense and complication of procedures involved, and the possible decrease in activity yields due to exposure of the enzyme to harsh environments or toxic reagents.

The selection of conditions for immobilization by covalent binding is more difficult than in any other carrier binding method. Ideally, the attachment of the enzyme to the carrier should involve only functional groups of the enzyme that are not essential for its catalytic action, and this is best achieved if the enzyme tertiary, primary and active site structures are known (Kennedy and Cabral 1983, 1987).

Kennedy and Cabral (1983) listed three main factors that have to be taken into account for the covalent immobilization of enzymes by a specific method. These are: (i) the functional group of the proteins suitable for covalent binding under mild conditions, (ii) the coupling reactions between the enzyme and the support, (iii) the functionalized supports suitable for enzyme immobilization. The functional groups of the proteins involved are amino, thiol, carboxyl, phenolic, guanidino, imidazole, disulfide, indole, thioether, and hydroxyl. Several types of coupling reactions exist and most of the common covalent coupling reactions involve coupling through protein amino groups, thiol groups, carboxyl groups, or aromatic rings of L-tyrosine and L-histidine. The

major classes of coupling reactions for the immobilization of enzymes are: diazotization, amide (peptide) bond formation, alkylation and arylation, Schiff's base formation, Ugi reaction, amidation reactions, thiol-disulfide interchange reactions, mercury-enzyme interactions, and γ-irradiation-induced coupling. Two of these coupling reactions, Schiff's base formation (discussed below under crosslinking method) and the diazotization, have found wide application with glass carriers (Haller 1983, Weetall 1976). The diazo-coupling method is based on the reaction of the enzyme with the aryldiazonium electrophilic groups of the carrier (Kennedy and Cabral 1987). A number of protein functional groups that can participate in azo coupling are: free amino groups, the histidine imidazole, and tyrosine phenolic groups. Some of the many different carrier materials that have been used in conjunction with the covalent binding method are: polysaccharides, acrylic polymers and copolymers, polyamides, proteins, and inorganic matrials.

Inorganic materials, such as alumina, bentonite, charcoal, nickel oxide, silicas, titanias and glass, have distinct advantages as carriers for immobilization of biologically active molecules (Filbert 1975). Generally, they have high mechanical strength, good resistance to solvent or microbial attack, support reusability, and are easy to handle. Controlled-pore glass (CPG), has additional and unique characteristics, particularly with reference to the precise control that may be exercised over its pore morphology (Filbert 1975, Haller 1983). The major reactive groups on porous glass are the silanol groups, and boron present on the surface. Because of both its surface hydroxyl groups and Lewis acid sites, porous glass exhibits a slight negative charge in aqueous media. These characteristics largely contribute to the phenomenon of strong adsorption of basic compounds on the glass surface (Filbert 1975, Haller 1983).

1.3.2.4 Crosslinking method.

The crosslinking immobilization method is based on the formation of covalent bonds between the enzyme molecules, by means of multifunctional reagents, leading to three-dimensional, crosslinked enzyme aggregates that are completely insoluble in water, without the use of water insoluble carriers (Kennedy and Cabral 1983, 1987). The most important advantage of this method is that one crosslinking reagent can be used to prepare, in a simple manner, immobilized enzymes that are almost pure protein (Zaborsky 1973). However, the insolubilization of the enzyme is critically dependent on a delicate balance of concentrations of the enzyme and crosslinking reagent, the pH and ionic strength of the solution, the temperature, and the time of the reaction. Some other disadvantages of this method are the large amount of enzyme required, the often unavoidable inactivation of enzyme caused by the participation of the active center on the crosslinkage, and the mechanical properties of the gelatinous nature of the enzyme derivatives (Kennedy and Cabral 1983). Among the considerable number of crosslinking agents described in the literature, glutaraldehyde has found widespread use for enzyme immobilization and is by far the most commonly used crosslinking reagent (Kennedy and Cabral 1983, Reichlin 1980).

1.3.2.5 Novel immobilization techniques.

Novel immobilization techniques are being proposed and some of them which involve modifications of established methodologies appear to be promising. Kobos *et al.* (1989) have recently reported the adsorption of suitably modified enzymes onto fluorocarbon surfaces. The protein ligands were controllably perfluoroalkylated before being introduced to the fluorocarbon matrix, resulting in an enhancement of protein avidity to the support, while biological activity and biospecificity were retained. Kilburn and co-workers (Ong *et al* . 1989), recently reported the immobilization of a

fusion protein to cellulose based matrix using the cellulose binding domain of *Cellulomonas fimi* exoglucanase. Despite the preliminary preparations of constructing and cloning the fusion gene that encodes the desired fusion protein, these authors believe that this technique offers a simple method of enzyme immobilization.

1.3.3 Characterization of immobilized enzymes.

Buchholz and Klein (1987) have listed guidelines for the characterization of immobilized biocatalysts, relevant to preparative and industrial applications. Their recommendations aim at raising the minimum level of characterization of all immobilized biocatalysts to an acceptable level so that adequate information for sensible comparisons and design purposes with a reasonable amount of effort can be obtained. Relevant topics are catalyst effectiveness and its optimization, inactivation mechanisms and operational stability, and more sophisticated reaction engineering concepts such as physical and mechanical parameters as well as transport phenomena and kinetics, which are intimately linked to biochemical systems. In order to properly characterize an immobilized enzyme system, basic chemical data on the carrier must also be known: basic chemical compounds (such as monomers), gross chemical composition (e.g., SiO_2), type of functional groups and their density, structure such as pore size and distribution, and specific surface. An accurate description of the immobilization procedure, as well as a knowledge of the immobilization efficiency, is crucial for the evaluation of the method and the biocatalyst in terms of reproducibility, scale up and economics.

1.4 Thesis objectives and rationale.

After a survey of the literature on peroxidases, it became apparent that, despite the potential of CPO as a commercial enzyme, very little work had been carried out to

study CPO under conditions that allow multiple uses of the same batch of enzyme. Some potential areas of study are: the methods of immobilization of CPO, kinetic studies with the immobilized enzyme, an assessment of the stability profiles of this enzyme under a variety of storage and operational conditions, effectiveness of the immobilized enzyme under optimum operational conditions.

The scope of this thesis project was to deal with some of these topics. The first area of the research project was to immobilize CPO, with an ultimate goal of testing the immobilized enzyme in the removal of phenolics from aqueous solutions. Towards this end, a preliminary evaluation of established enzyme immobilization techniques and immobilization carriers was to be conducted to select a suitable method and support material for CPO immobilization. If need be, the selected method was to be modified to achieve optimum coupling yields and stability of immobilized enzyme, or a novel method was to be developed.

Another area of research was the evaluation of existing CPO assays to select a suitable and sensitive assay system for the quantitation of the immobilized enzyme. Because of the changed properties of the enzyme in the immobilized form, standard enzyme assays used to measure activity of soluble CPO may not be suitably applied to the immobilized enzyme (Mattiasson and Mosbach 1976). Therefore modifications of a selected established enzyme assay were to be carried out or a novel assay was to be developed for the immobilized enzyme.

After a successful immobilization of CPO to a suitable carrier, the immobilized enzyme system was to be characterized. This characterization would consist of a comparative study of the properties of the soluble and immobilized enzymes, with respect to kinetic constants, storage and operational stabilities.

The last area of study would involve the application of immobilized CPO in the removal of phenol from aqueous solution. This study is part of a series of preliminary

work from our laboratory which consists of testing and evaluating the efficiency of CPO in the treatment of industrial wastewaters containing phenolic compounds. Initial studies were carried out with the soluble enzyme by Carmichael (1987) as part of his M. Sc. thesis project. The objective of this study greatly influences the choice of the immobilization carrier and method. A suitable carrier must possess (i) good mechanical strength and (ii) inertness to microbial degradation, which are the basic properties of rigid inorganic carriers, such as glass particles. The immobilization method of choice must provide a stable immobilized enzyme derivative. Therefore a method resulting in the covalent binding of CPO to glass would be appropriate for the projected immobilized enzyme system.

2. MATERIALS AND METHODS.

2.1 Microbiological.

The fungal strain, *Caldariomyces fumago* CMI 89362, was obtained from the Commonwealth Mycological Institute, Kew, Surrey, England. For long term storage, the organism was maintained on potato dextrose agar plates at 4°C and transferred to fresh plates every 6 months (Pickard 1981, Carmichael 1987).

2.2 Carriers and chemicals.

The sources of the chemicals used were as follows: glutaraldehyde (25%), 3-aminopropyltriethoxysilane (APES), *o*-phenylenediamine, *p*-phenylenediamine, *o*-dianisidine, and N,N,N',N'-tetramethyl-*p*-phenylenediamine (TMPD) from Sigma chemical company. Analytical grade phenol, obtained from BDH chemicals, was used without further purification. Hydrogen peroxide (30%) was obtained from AMACHEM. All other chemicals were of reagent grade. The porous carriers, 200-400-mesh size, with the following pore sizes: 75, 240 and 546Å, for non-derivatized glass beads, CPG-75, CPG-240, and CPG 546, respectively, and 170Å for aminopropyl-glass, A-CPG-170, were from Sigma. The non-porous glass, the 40-mesh glass, and the 106 microns and finer glass, were from BDH Chemical Ltd. and Sigma, respectively.

2.3 Purification of chloroperoxidase.

Chloroperoxidase (CPO), kindly donated by Dr. M.A. Pickard (Microbiology Department, U of A), was produced from *C. fumago* inoculum grown in a phytone-fructose salts medium (Carmichael 1987, Pickard 1981). Two types of fermenters (1.0-liter airlift and 50-liter LH 2000 Series fermenter) were used for the

semi-continuous production of the enzyme (Carmichael and Pickard 1989). The 50-liter fermenter had a temperature and pH PID (proportional, integral, derivative) type controllers, and a pO_2 proportional type controller. CPO produced was harvested from the culture supernatant and stored at -20°C until purified. A portion of this material was used in these studies.

CPO was purified from *C. fumago* culture supernatant as previously described by Pickard and Hashimoto (1982), with slight modifications (Carmichael 1987). The four purification steps involved the concentration of the fungal growth medium in a Millipore Pellicon tangential flow cassette system; the precipitation of the pigment with polyethylene glycol; the removal of polyethylene glycol by dialysis in a Millipore Pellicon tangential flow cassette system; and finally, further purification of CPO by ion-exchange chromatography on a DE-52 cellulose column. The purified enzyme had a typical Reinheitzahl coefficient (Rz) value of 1.4 or greater, indicating a purity of 95% or greater (Morris and Hager 1966). The purified enzyme preparation contained two isoenzymes, with pI values of 3.6 and 3.9 (Pickard and Hashimoto 1982, Sae and Cunningham 1979).

2.4 Development of the colorimetric peroxidase assay: spectral analysis of the oxidation products.

The aromatic amines, *o*-phenylenediamine, *p*-phenylenediamine, *o*-dianisidine, and TMPD, were oxidized by CPO in the presence of H_2O_2. The spectra of the oxidation reaction products were obtained by scanning reaction mixtures in a PYE-UNICAM model SP8-100 UV-visible spectrophotometer. The relative absorbance of the oxidized substrates was determined at their λ_{max}.

2.5 Methodology of CPO immobilization on glass.

CPO was immobilized on glass particles initially according to the glutaraldehyde linker method the methodology described by Weetall (1976). The procedure consisted of three major steps: (i) the amination (or silanization) of acid-washed glass, (ii) the coupling of a glutaraldehyde linker to the aminopropyl-glass, and finally (iii) the immobilization of CPO onto the derivatized support material (Figure 2.1). Washing the glass beads between the steps was carried out either by decantation or on a sintered glass filter.

2.5.1 Glutaraldehyde linker method.

In the glutaraldehyde linker method, the alkylamine glass was initially reacted with glutaraldehyde to form a linker arm before binding CPO to the glass. Each experimental condition was carried out at least in duplicate.

2.5.1.1 Amination of glass beads.

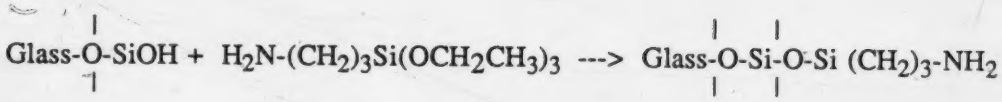
The glass particles were washed in 10% nitric acid at 80-90°C, for 1 hour. The glass was then extensively washed with glass-distilled water or Milli Q water. This treatment both cleans the glass beads and maximizes the amination reaction. Three procedures were followed to aminate the glass. In the first procedure (Olsson and Ögren 1983, Weetall 1976) the acid-washed glass was reacted for 3 hours with a 10% aqueous solution of APES, pH 3.45, at 70°C. The silane solution (20 ml) was added to 1 g of glass. The aminopropyl-glass was washed extensively with water, air dried, and further dried overnight in oven at 95-100°C. The aminated glass was again washed with water before the next treatment.

In the other two procedures (Olsson and Ögren 1983, Weetall 1976), the amination of acid-washed glass was carried out in an organic silane solution. In one of

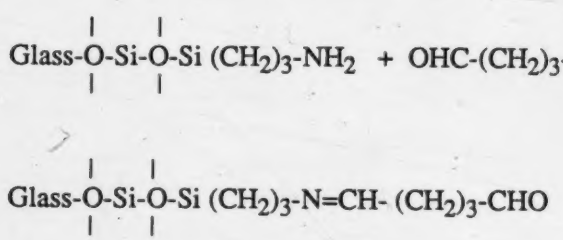
Figure 2.1. The three major steps of the glutaraldehyde linker method of enzyme immobilization.

Shown are the chemical groups involved in the interaction between (i) glass and aminopropyltriethoxysilane, (ii) aminopropyl- (alkylamine or aminated) glass and glutaraldehyde, and (iii) glutaraldehyde-treated aminopropyl-glass and enzyme to prepare an immobilized enzyme adduct.

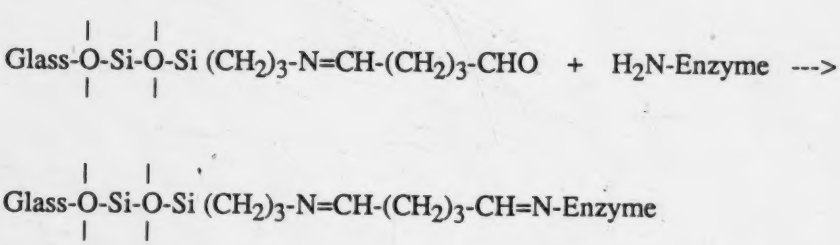
(i) Glass amination step.



(ii) Glutaraldehyde linking reaction step.



(iii) Enzyme immobilization reaction step.



the procedures, 50 ml of 10% APES solution in toluene were added to 1 g of glass and refluxed for 3 hours. The aminopropyl-glass was first washed with acetone, then washed extensively with water, and dried. The third procedure involved the evaporation of the solvent to dryness with forced air. To 1 g of glass was added 25 ml of 1% APES solution in acetone. The acetone was then evaporated with forced air, leaving a yellowish patchy layer of APES on the glass. At the end of the treatment with APES, the aminated glass was washed with acetone and water, and finally dried.

2.5.1.2 Glutaraldehyde treatment of aminated glass.

Aminated glass (200 mg) was treated with 1 ml of 2.5% glutaraldehyde solution in 100 mM phosphate buffer, pH 7.0, for 1 hour at room temperature. The reaction was allowed to proceed first under reduced pressure to remove air from the pores of the carrier and maximize the interaction of the reagent with the carrier surface (Haller 1983). In the optimization studies of the glutaraldehyde treatment step, the standard reaction time was 1 hour, except in the study of the effect of the reaction time on CPO immobilization, where time was the variable parameter. All the studies were carried out at room temperature and duplicate samples of 40-mesh glass beads (100 mg) were used in small screw-capped vials. At the end of the specified time, the reaction was stopped by washing the glass particles with water.

2.5.1.3 CPO immobilization on glutaraldehyde-treated glass.

In the initial experiments, the enzyme was immobilized on aminopropyl-glass previously treated with glutaraldehyde. To 100 mg of 40-mesh glass was added 500 µl of concentrated CPO solution (6.7 mg/ml in 100 mM phosphate buffer, pH 5.5). The reaction was carried out overnight at 5°C. At the end of the reaction, the immobilized enzyme derivative was extensively washed first with 500 mM and then 100 mM

phosphate buffers, pH 5.5, to remove any enzyme loosely bound to the support.

2.5.2 Glutaraldehyde crosslinking method.

Because the glutaraldehyde linker method gave only poor results, a novel method was developed. CPO was directly immobilized on aminopropyl-glass, followed by the crosslinking of the glass-bound enzyme with glutaraldehyde. The porous glass beads were generally used in these studies because the extent of the immobilization reaction could be easily vizualized by the coloration of the aminated glass into red beads, due to the heme of the enzyme. As was the case with the glutaraldehyde linker method, each experimental condition was carried out at least in duplicate.

2.5.2.1 CPO immobilization on alkylamine glass.

The immobilization reaction was carried out at room temperature for 4 hours, with occasional shaking. The effect of enzyme concentration on loading was determined by presenting different amounts of CPO to 20 mg of glass, in 500 µl of 2 mM buffer, pH 6.0. At the end of the reaction, the immobilized enzyme preparation was washed extensively with 2 mM buffer, pH 6.0, before reacting it with glutaraldehyde. The amount of enzyme not retained on the carrier in this step was determined from the washings.

2.5.2.2 Glutaraldehyde crosslinking of glass-bound CPO.

To 20 mg of immobilized enzyme derivative was added 500 µl of 2.5% glutaraldehyde solution in 100 mM phosphate buffer, pH 6.0. The reaction was carried out at room temperature for 30 minutes. At the end of the reaction, the immobilized enzyme derivative was washed with 2 mM buffer, pH 6.0, and the enzyme released during this step was determined from the washings. To determine the amount of

enzyme covalently bound to the glass, the immobilized enzyme derivative was finally washed with a buffer of high concentration (500 mM, pH 5.5 or 300 mM, pH 4.0).

2.6 Quantitation of immobilized CPO.

The amount of CPO bound to the carrier was determined by two different approaches. In the first approach, the amount of immobilized CPO was determined indirectly from the difference of enzyme presented to the carrier and the enzyme collected in the washings throughout the course of the immobilization procedures. This was termed as the "difference method" of determining the amount of bound enzyme. In the second approach, the amount of immobilized CPO was directly determined (i) from the activity of the immobilized enzyme derivative, based on the specific activity of the soluble enzyme, and (ii) from the amount of protein resolubilized from the carrier by alkaline hydrolysis, using the modified Lowry assay. For routine analysis, the A_{403} value (a measure of the heme content) was used to quantitate the amount of immobilized CPO by the "difference approach", after a correlation was established between the A_{403} values and the amount of protein determined by the modified Lowry assay.

2.7 Enzyme assays.

The activity of a CPO preparation was determined by measuring the chlorination or the oxidation activity of the enzyme.

2.7.1 Chlorination assay.

The activity of the soluble enzyme was determined by measuring the initial rate of the monochlorodimedone chlorination reaction (Morris and Hager 1966) as modified by Pickard (1981). Reaction mixture contained 100 µl of potassium phosphate buffer,

pH 2.75, 20 µmol of potassium chloride, 2 µmol of hydrogen peroxide, 0.1 µmol of monochlorodimedome, and CPO in a volume of 1.0 ml. The reaction was started with the addition of hydrogen peroxide to the reaction mixture, and the disappearance of absorbance at 278 nm was monitored using a PYE-UNICAM SP8-100 double-beam spectrophotometer, at 30°C. One enzyme unit was defined as the amount of CPO that causes the formation of 1 µmol of dichlorodimedone under standard conditions (Morris and Hager 1966).

2.7.2 Oxidation assay.

Routinely, the activity of CPO was determined by a colorimetric assay based on the catalytic oxidation of TMPD in the presence of H_2O_2. The reaction was carried out at room temperature for 5 minutes. The reaction mixture (1 ml) contained the enzyme, 20 µmol TMPD, 1 µmol hydrogen peroxide, and 100 µmol potassium phosphate buffer, pH 6.0. The reaction was quenched with cold acid-methanol mixture, and the absorbance read at 563 nm. The activity of the immobilized enzyme was determined by adding the reactants, in final concentrations of 1 mM hydrogen peroxide and 20 mM TMPD, to the buffer-washed immobilized CPO preparation. The reaction mixture volume was varied depending on the activity of the immobilized enzyme derivative, so that the A_{563} value of a 20-fold dilution of the reaction products in the stopping reagent would not exceed 1.0 A_{563} unit. The controls contained immobilized enzyme derivative inactivated during a 10-minute heat treatment at 70°C, or the reaction mixture without the enzyme. The reaction was carried out with shaking at room temperature for 5 minutes. One µg of CPO (Lowry protein) caused an increase of 40 A_{563} units in a 5-minute reaction, at pH 6.0.

The enzyme specific activity of 1660 µmol dichlorodimedome formed/minute/

mg of crystalline CPO, determined from the standard chlorination assay (Morris and Hager 1966), was used to determine the relationship between the two assays.

2.8 Protein assay.

The Lowry procedure for the estimation of proteins (Lowry *et al.* 1951) was modified to increase the concentration of NaOH and ensure the complete hydrolysis of the immobilized enzyme. The glass-immobilized enzyme was incubated in 400 µl of 1 N NaOH for 30 minutes at 37°C. At the end of the alkaline hydrolysis treatment, 800 µl of glass distilled (or milli Q) water and 1 ml of 2 times concentrated copper reagent were added to the samples. The mixture was incubated at room temperature for 30 minutes, before the addition of 200 µl of 1 N Folin-Ciocalteau reagent, prepared in 1 N HCl to neutralize the base. The mixture was again incubated for 30 minutes, and the absorbance at 750 nm was measured in a spectrophotometer. The protein standards BSA and soluble CPO were treated in the same manner as the immobilized enzyme.

2.9 Analysis of phenol oxidation.

A quantitative analysis of phenol remaining in the reaction mixture was carried out by gas chromatography (GC). Aliquots (5 µl) of the reaction mixture were injected in a Hewlett-Packard model 5790 GC equipped with a flame ionization detector. The separation column was a 2m x 2mm stainless steel column, packed with 5% polyphenyl ether coated Tenax-GC (60-80-mesh). The carrier gas was nitrogen flowing at the rate of 30 ml/minute. With the use of phenol standard solutions, the phenol peak was detected at a retention time of 2.80 minutes. The peak area was determined using a Hewlett-Packard model 3390A integrator. The GC analysis was performed at a constant temperature of 200°C. The injector and detector temperatures were 230 and 250°C, respectively.

3. RESULTS.

3.1 CHLOROPEROXIDASE ASSAY.

The primary goals of this work involved (i) the evaluation of strategies for the immobilization of chloroperoxidase (CPO) on rigid supports, such as glass beads or sand, (ii) the characterization of this immobilized enzyme system, and (iii) the application of the immobilized enzyme in the oxidative conversion of phenol into precipitable polymers. An important preliminary step in this project was to find or develop a highly sensitive and rapid enzyme assay to use on a routine basis for the quantitation of immobilized CPO.

The most commonly used assay for CPO, described by Morris and Hager (1966) is based on the kinetic measurement of the initial chlorination rates, and is designed for a soluble enzyme system where homogeneous conditions can be maintained during the course of the assay. However, when CPO is immobilized on insoluble carriers, such as controlled-pore glass (CPG) beads, homogeneous conditions could not be maintained without a mixing device. Such a mixing mechanism system, required to ensure a maximum contact between the insoluble enzyme and its substrate, was difficult to achieve with the equipment on hand. An additional problem encountered with this assay system was the interference of the beads with the absorbance readings. A possible solution to this problem was to carry the reaction with shaking outside the spectrophotometer, and rapidly take samples out to determine the initial rates at very short intervals. This proved to be a very tedious procedure, and the reproducibility of the data was also compromised as a result of multiple mechanical handling of the samples.

Therefore one of the first studies involved the development of a colorimetric

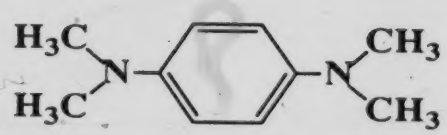
assay for the measurement of activity of soluble CPO. This new assay was intended to be used routinely in the quantitation of immobilized CPO. For a colorimetric assay would permit both rapid detection of immobilized enzyme and quantitative determinations of bound activity.

3.1.1 Spectral characteristics of the assay substrate.

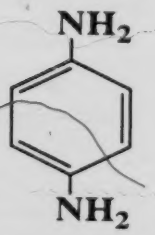
The first step in the development of the enzyme assay was to select a substrate for the assay. This selection was based primarily on the ability of the substrate compound to develop a detectable color when oxidized by CPO. In the presence of H_2O_2, CPO was shown to carry out the oxidation of a number of aromatic compounds, resulting in the formation of colored products (Carmichael et al. 1985, Corbett and Chipko 1979, Griffin and Ashley 1984, Neidleman and Geigert 1986).

Four aromatic amines were tested as suitable substrates for the calorimetric assay for CPO (Figure 3.1). The catalytic oxidation of tetramethyl-*p*-phenylenediamine (TMPD), *o*-phenylenediamine and *p*-phenylenediamine, and *o*-dianisidine resulted in the formation of blue, yellow, and reddish brown colored products, respectively. The absorption spectra of the colored products obtained in the visible region showed two peaks for TMPD, with λ_{max} at 563 and 610 nm (Figure 3.2). It was observed that during prolonged incubation period at room temperature, the peak at 563 nm was much more stable than that at 610 nm. Therefore, absorbance readings of TMPD oxidation were carried out at 563 nm. The oxidation of the other three substrates, *o*-dianisidine, *o*-phenylenediamine, and *p*-phenylenediamine, gave rise to spectra with single peaks in the visible region, λ_{max} 460, 440 and 463 nm, respectively.

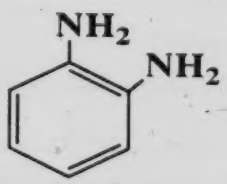
Figure 3.1. Chemical structures of the aromatic amines tested as suitable chromogens for the colorimetric chloroperoxidase assay.



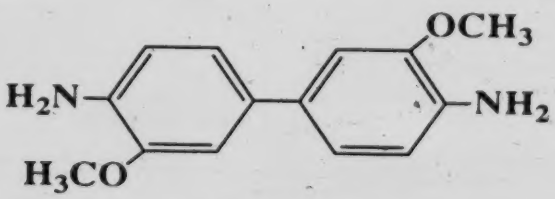
N,N,N',N'-tetramethyl-*p*-phenylenediamine



p-phenylenediamine



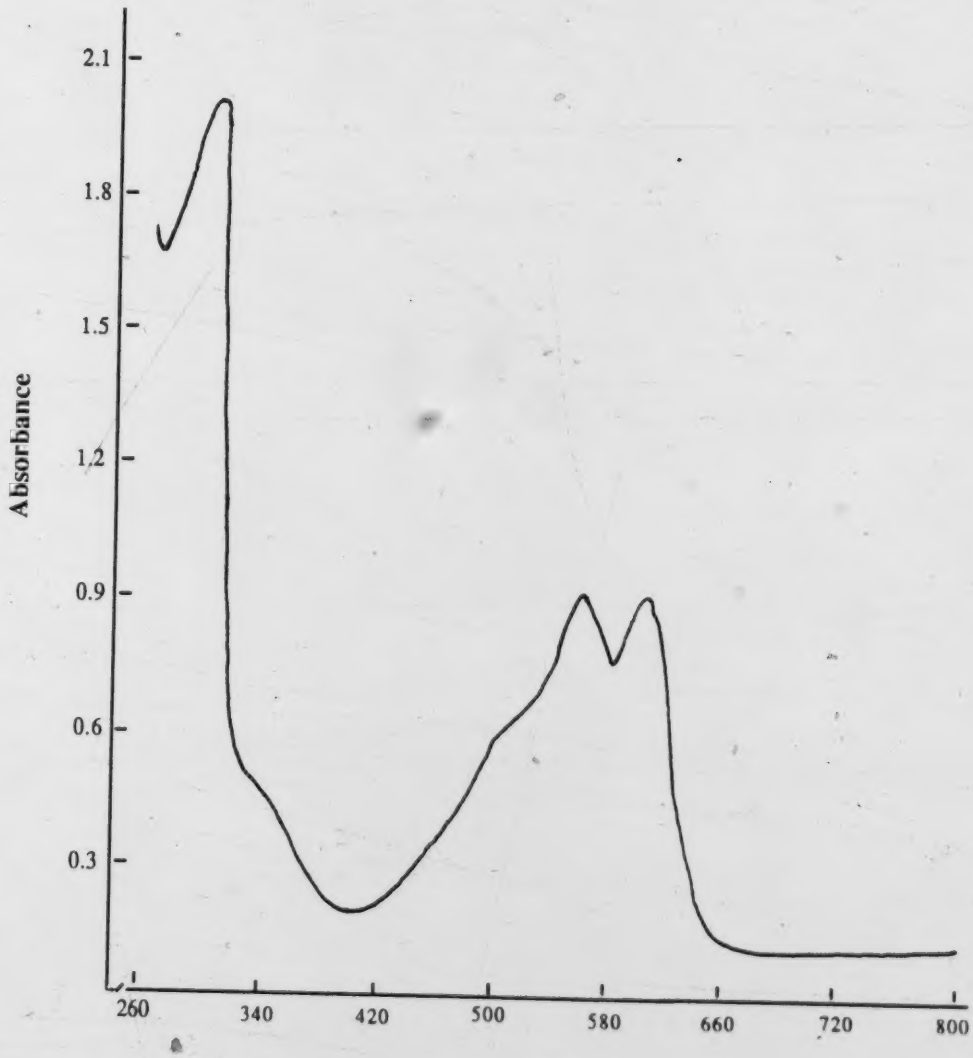
o-phenylenediamine



o-dianisidine

Figure 3.2. Absorption spectrum of oxidized N,N,N',N'-tetramethyl-*p*-phenylenediamine (TMPD).

The TMPD oxidation reaction was carried out at pH 2.75, in 100 mM potassium phosphate buffer, in the presence of hydrogen peroxide and CPO. The absorption spectrum of the oxidation product was scanned in a PYE-UNICAM model SP8-100 UV-visible spectrophotometer.


2.1
1.8
1.5
1.2
0.9
0.6
0.3
Absorbance
260
340
420
500
580
660
720
800
Wavelength (nm).

3.1.2 Evaluation of the substrate for the assay.

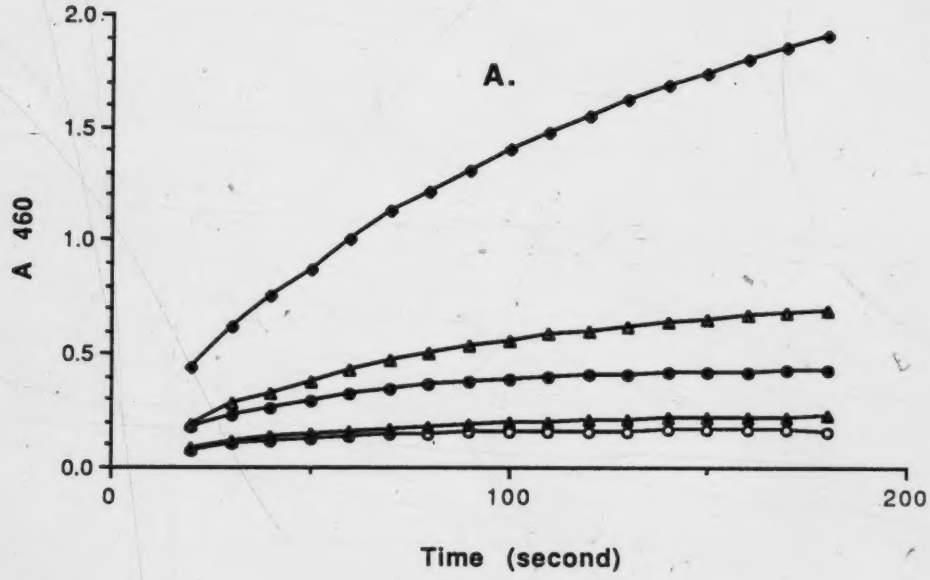
To evaluate the substrate for the assay, two important properties of its oxidation product were considered: (i) the stability of the colored reaction product or the colored reaction intermediate species, and (ii) the relative intensity of the developed color. The latter was particularly important for the development of a more sensitive assay.

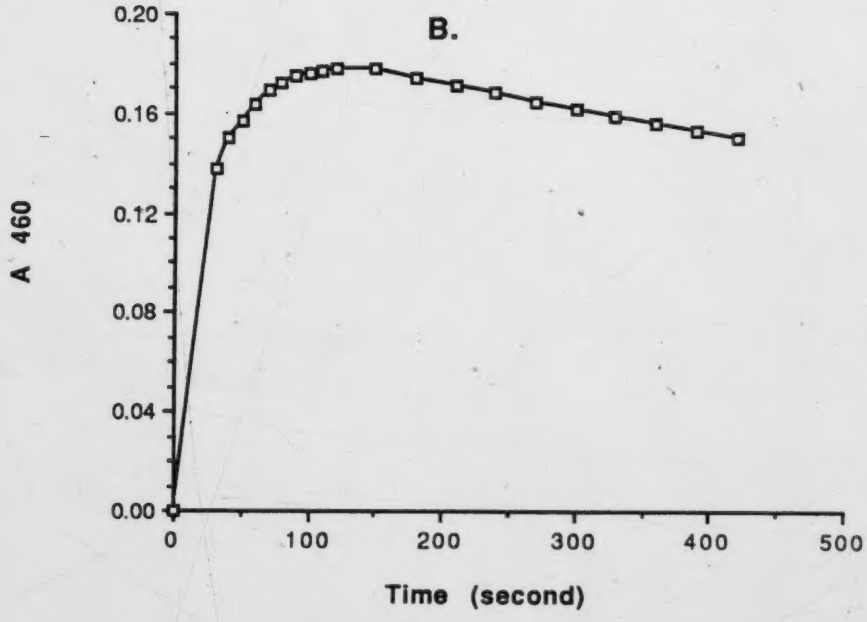
Solutions of aromatic amines *o*-dianisidine, *o*-phenylenediamine, and *p*-phenylenediamine and TMPD, were freshly prepared daily, and kept cold during use. The solubility of these compounds in water was very limited. Therefore, to obtain concentrated stock solution, a mixture of methanol and 100 mM H_3PO_4/potassium phosphate buffer, pH 2.75 (1:1) was used as solvent. However, the presence of methanol in the reaction mixture affected the enzyme activity, and this effect was dependent on the final concentration of methanol. For this reason, the substrates prepared in the buffer/methanol mixture were only used in studies comparing the four substrate compounds.

The first substrate investigated was *o*-dianisidine. This compound has been used as a chromogen in peroxidase colorimetric assay (Worthington 1972). Therefore, initial studies were carried out to define the conditions under which this substrate could be used in an assay for immobilized CPO. When the soluble enzyme was assayed by this method, the results indicated no linear relationship between the amount of CPO and the color formation (Figure 3.3A). To evaluate the stability of the developed color, a lower enzyme concentration was used. Under these conditions it was observed that the color formation increased with time, up to a maximum before decaying (Figure 3.3B). This indicated that the developed color was probably associated with a short-lived reaction intermediate species, which was rapidly converted to an uncolored product, or to another colored product with a different λ_{max}. Higher enzyme concentration

Figure 3.3. Oxidation of *o*-dianisidine by CPO.

A. Effect of enzyme amount on the formation of the colored product. Different amounts of enzyme [13 ng (o), 17 ng (▲), 22 ng (●), 33 ng (Δ) and 67 ng (♦) CPO], were added to a 3-ml reaction mixture containing: 2.4 µmol H_2O_2, 1.5 µmol *o*-dianisidine, and 150 µmol phosphate buffer, pH 5.5. The reaction was carried out at room temperature and the absorbance recorded over time. **B**. Effect of reaction time on the formation and decay of the colored product at low enzyme amount (13 ng CPO). The reaction mixture was as described in **A**.


A.
A 460
2.0
1.5
1.0
0.5
0.0
0
100
200
Time (second)


B.
A 460
0.20
0.16
0.12
0.08
0.04
0.00
0
100
200
300
400
500
Time (second)

appeared to mask this observation or delay its occurrence.

Since the data from the study with *o*-dianisidine were not satisfactory, other compounds were investigated. To analyze the relative intensity and stability of the color developed from the oxidation of the other three aromatic amines, different amounts of each substrate were presented to the enzyme. After a 2-minute reaction, the absorbance was recorded at their respective λ_{max}. The results are shown in Table 3.1. At 5 mM, the oxidation products of TMPD had an absorbance of 1.93 units. The color produced from TMPD oxidation was 3 and 4 times more intense than that produced from the oxidation of *p*-phenylenediamine and *o*-phenylenediamine, respectively. A time course experiment indicated an increase in the color intensity with time. As with *o*-dianisidine, the intensity of the color decreased for the phenylenediamines after reaching a maximum value. However, during the oxidation of TMPD under the same reaction conditions, there was no detection of a maximum A_{563} value or a decrease in the color intensity.

On the basis of these results and the reported health hazard associated with the use of the other compounds (Worthington 1988), TMPD was selected as the substrate for the assay. A disadvantage in using TMPD was that it autooxidized in the presence of air. Further studies were carried out with this compound to define the conditions of the assay. A more concentrated aqueous solution of TMPD, obtained from TMPD-$(HCl)_2$, the salt derivative of this compound, was used in these studies.

3.1.3 Characterization of the assay.

The characterization of the assay was carried out to determine the effects of the reaction mixture, enzyme amount, and reaction time on CPO activity.

Table 3.1. The relative intensity of the colored products formed during the hydrogen peroxide-dependent oxidation of *p*-phenylenediamine, *o*-phenylenediamine, and TMPD by CPO.

Substrate	λ_{max}[(1)]	Substrate concentration (mM)			
		5.00	3.75	2.50	1.25
p-phenylenediamine	A_{463}	0.59	0.51	0.39	0.25
o-phenylenediamine	A_{440}	0.46	0.32	0.19	0.07
TMPD	A_{563}	1.93	1.73	1.35	0.79

(1) The color intensity of each oxidized substrate was determined colorimetrically at their corresponding λ_{max}, after a 2-minute reaction. The reaction mixtures contained hydrogen peroxide (2 µmol), CPO (2.6 ng) in 1 ml 100 mM phosphate buffer, pH 2.75.

3.1.3.1 Quenching the reaction.

An observation made during the evaluation of the substrate compounds was that the simple exposure of an aqueous solution of TMPD to air resulted in its oxidation. Therefore, prior to characterizing the colorimetric assay, attempts were made to stop the reaction after a specified time, and stabilize the color. This required both the inactivation of the enzyme and the decrease of the autooxidation rate of TMPD. Using reaction mixtures in which TMPD oxidation had reached an A_{563} of 1.0 to 1.5, the effect of different quenching mixtures was investigated. The effect of each reagent on the final A_{563} value, recorded at the end of the enzyme-catalyzed reaction, was analyzed to determine the efficiency of the different reagents in quenching the reaction. A good quenching reagent was expected to cause no change in the final A_{563} value, other than changes resulting from the dilution of the reaction mixture.

In an initial attempt, acid and base solutions were added to the reaction mixture in a final concentration of 0.1 to 0.2 mM, and the change in the final A_{563} was recorded thereafter for a period of time. Acids (HCl, H_3PO_4, H_2SO_4) and bases (NaOH, KOH) not only inactivated the enzyme, but also caused the decay of the developed color. Therefore these reagents were not investigated further.

The other reagents analyzed were hydrogen peroxide and methanol. When H_2O_2 and methanol were used in final concentrations of 30 mM and 50%, respectively, a partial quenching of the reaction was observed. Unlike the acids and bases, these reagents did not cause the decay of the color. However, a slow increase in color intensity resulting from the autooxidation of TMPD was observed. This autooxidation rate was found to increase with both pH and temperature. Since methanol was not a substrate for CPO, this reagent was preferably selected over H_2O_2 as the reaction

stopping reagent.

After several mixtures were investigated, a suitable stopping reagent was found to be methanol/H_3PO_4 mixture. Methanol was mixed with concentrated H_3PO_4 to adjust the pH to about 1.8, and the mixture was kept cold during use. This cold methanol-acid reagent stopped the enzyme-catalyzed reaction and reduced considerably the autooxidation rate of TMPD (Figure 3.4). By lowering the pH of methanol solution with phosphoric acid, a two-fold decrease in TMPD autooxidation rate was achieved. A further decrease was obtained by keeping both the reagent and the quenched reaction mixture in an ice bath, which also minimized the evaporation of methanol, providing more consistant A_{563} values. Therefore, in the future studies, the stock solutions of TMPD were kept frozen at -20°C until use.

3.1.3.2 Effect of the substrate concentration on CPO activity.

The activity of the enzyme increased with H_2O_2 concentration to a maximum activity at about 1 mM H_2O_2. Then a decline in activity was observed with further increase in H_2O_2 concentration (Figure 3.5A). The results appeared to indicate the inactivation of the enzyme or the classical phenomenon of substrate inhibition at high H_2O_2 concentrations. The maximum velocity observed was presumably the product of combined effects of the substrate inhibition and increase in the catalytic rate. The curve obtained by varying the concentration of TMPD indicated that the enzyme active sites were saturated at higher concentrations of this substrate (Figure 3.5B). The effect of the ionic strength on CPO activity was also investigated at pH 2.75. The results indicated that the phosphate buffer concentration affected the activity of the enzyme at 400 mM, and a progressive decrease in activity was observed as the concentration was further

Figure 3.4. Effect of quenching mixtures on the stability of the color from TMPD oxidation.

The peroxide-dependent oxidation of TMPD by CPO was carried out at room temperature, in a 1-ml reaction mixture containing: 2 μmol hydrogen peroxide, 5 μmol TMPD, 20 ng CPO and 100 μmol phosphate buffer, pH 2.75. The reaction was allowed to proceed until an absorbance value of more than 1.0-1.5 A_{563} units was reached. The reaction was then quenched with 1 volume of methanol at room temperature (o), methanol at 10°C (●), methanol/H_3PO_4 mixture at room temperature (Δ), and methanol/H_3PO_4 mixture at 10°C (♦). And the change in the absorbance was monitored at 563 nm. The spectrophotometer temperature was set at 14°C.

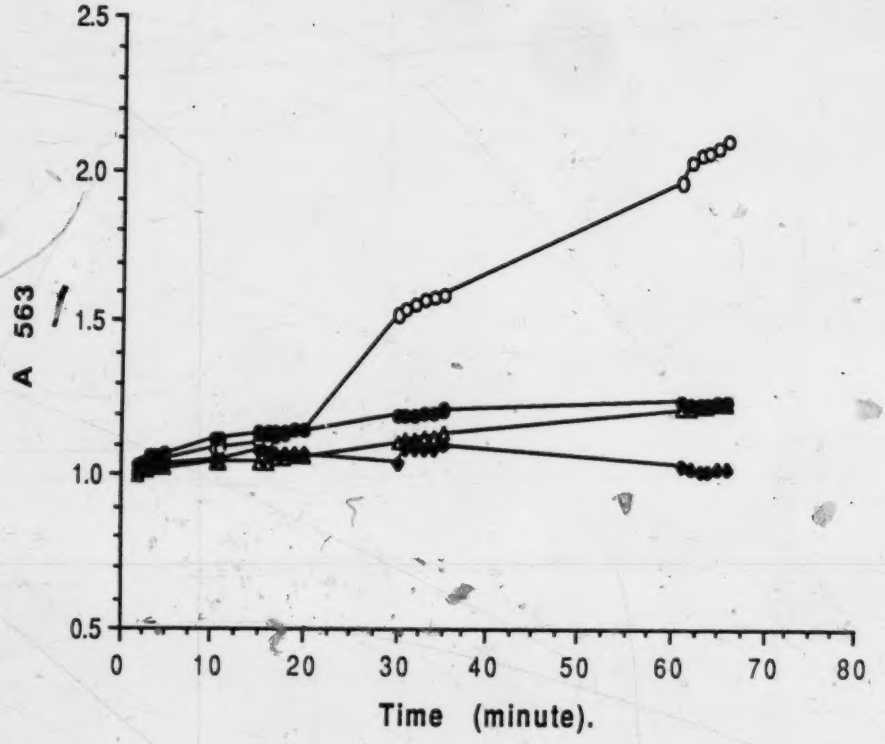
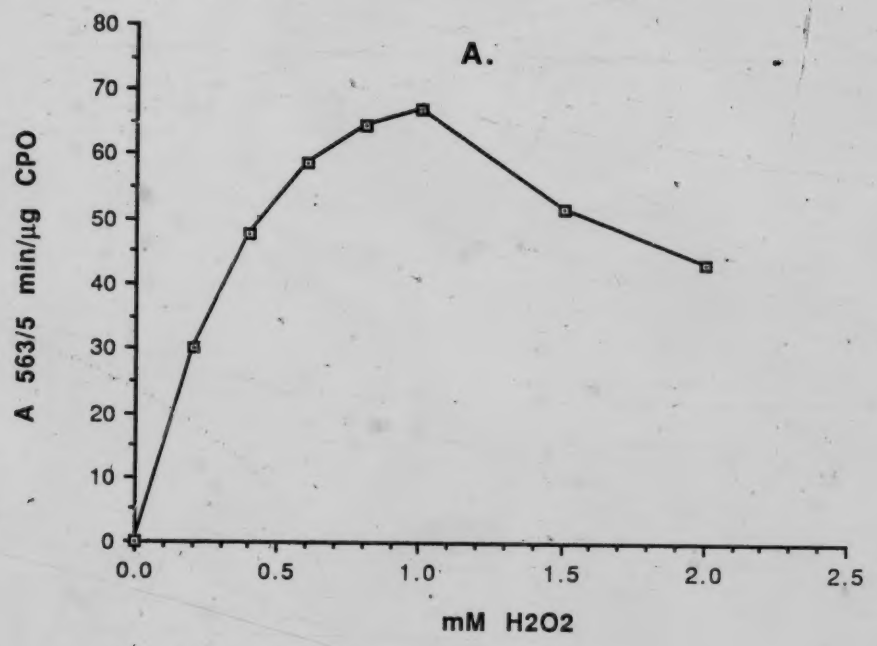
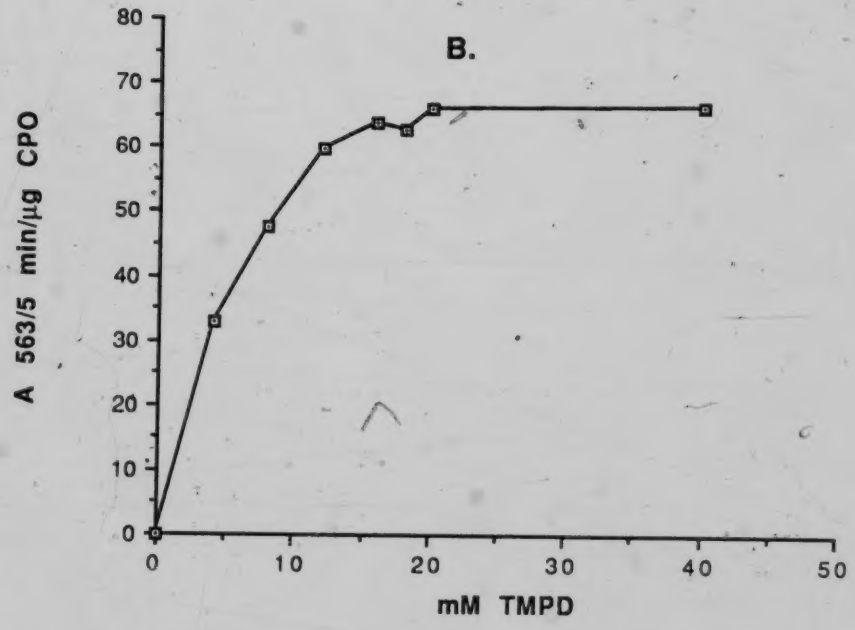

2.5
2.0
1.5
1.0
0.5
A 563
0
10
20
30
40
50
60
70
80
Time (minute).

Figure 3.5. Effects of substrate concentration on the catalytic velocity of TMPD oxidation by CPO.

The reaction was carried out at room temperature for 5 minutes in 100 mM phosphate buffer, pH 2.75. **A**. The effect of hydrogen peroxide was monitored by measuring the initial rates of TMPD oxidation as the concentration of hydrogen peroxide was varied in a 1-ml reaction mixture, at constant amount of TMPD (20 µmol) and CPO (20 ng). **B**. The effect of TMPD was monitored by measuring the initial rates of TMPD oxidation as the concentration of TMPD was varied in a 1-ml reaction mixture, at constant amount of hydrogen peroxide (1 µmol) and CPO (20 ng). Points displayed are means of two determinations.


A.
A 563/5 min/µg CPO
0
10
20
30
40
50
60
70
80
0.0
0.5
1.0
1.5
2.0
2.5
mM H2O2


B.
A 563/5 min/µg CPO
0
10
20
30
40
50
60
70
80
0
10
20
30
40
50
mM TMPD

increased (Table 3.2).

Based on these results, the standard assay mixture used in the quantitation of CPO activity was defined as follows: 1 mM H_2O_2 and 20 mM TMPD in 100 mM potassium phosphate buffer. Although similar substrate concentration effects were observed at pH 2.75, 5.5, and 6.0, the pH 6.0 was chosen for the assay, because of greater stability demonstrated by the immobilized enzyme at this pH, as will be discussed later.

3.1.3.3 Relationship between product formation and enzyme amount.

The enzyme concentration range, for which a linear relationship between product formation and enzyme amount is observed, was determined by varying the amount of enzyme in the reaction mixture in the standard 5-minute reaction. A linear relationship was obtained at CPO amount ranging from 13 ng to 1.6 μg (Figure 3.6). This linear response was also observed over a period of 10 minutes, the minimum reaction time tested. During the reaction time study, three different amounts of enzyme (67, 134, and 201 ng CPO) were tested (Figure 3.7). Kedderis et al. (1980) also reported a similar broad linear response of product formation with respect to CPO concentration (up to 1.8 μg of enzyme) during the oxidation of N,N-dimethylaniline.

3.2 CHLOROPEROXIDASE IMMOBILIZATION.

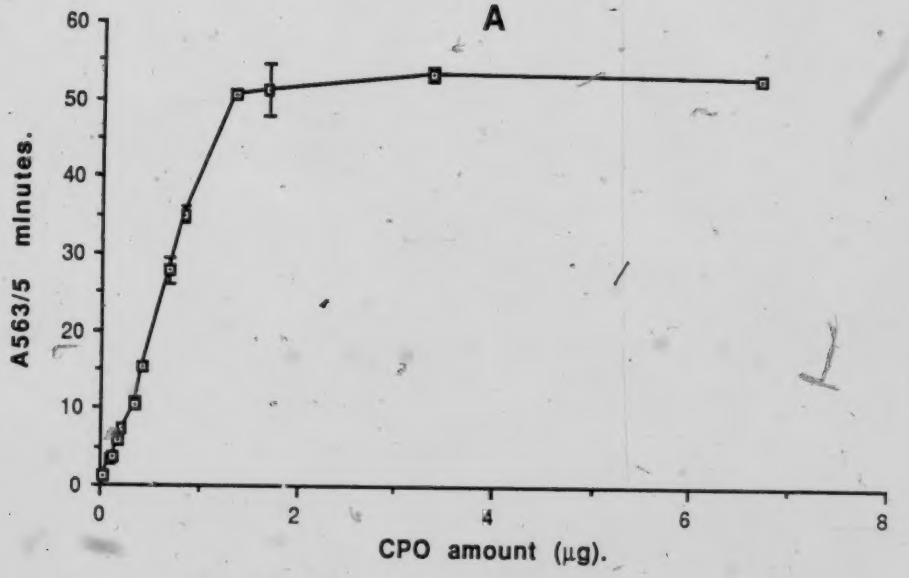
A major objective of this research was to immobilize CPO, and test the potential of the immobilized enzyme derivative in the oxidation of phenol. Before immobilizing CPO, a decision was made in choosing (i) the immobilization carrier and (ii) the immobilization method. Because the proposed application of the immobilized

Table 3.2. Effect of phosphate buffer concentration on colorimetric CPO assay.

Phosphate buffer, pH 2.75 concentration (mM)	CPO activity (A_{563}/5 min/μgCPO)
50	40.4
100	39.5
200	39.8
300	37.6
400	32.9
500	31.8
600	29.2
700	28.7
800	28.8
900	16.0
1000	14.0

Figure 3.6. Dependence of TMPD oxidation rate on CPO amount.

The relationship was studied in a 1-ml reaction mixture containing 1 μmol H_2O_2, 20 μmol TMPD, CPO, and 100 μmol phosphate buffer, pH 6.0. The reaction was carried out at room temperature for 5 minutes. Points displayed are means ± SD from 4 determinations. To show the best fit line to points in (**A**), the data points at high enzyme amounts, outside the linear region, are omitted in (**B**).


A
A563/5 minutes.
60
50
40
30
20
10
0
0
2
4
6
8
CPO amount (µg).

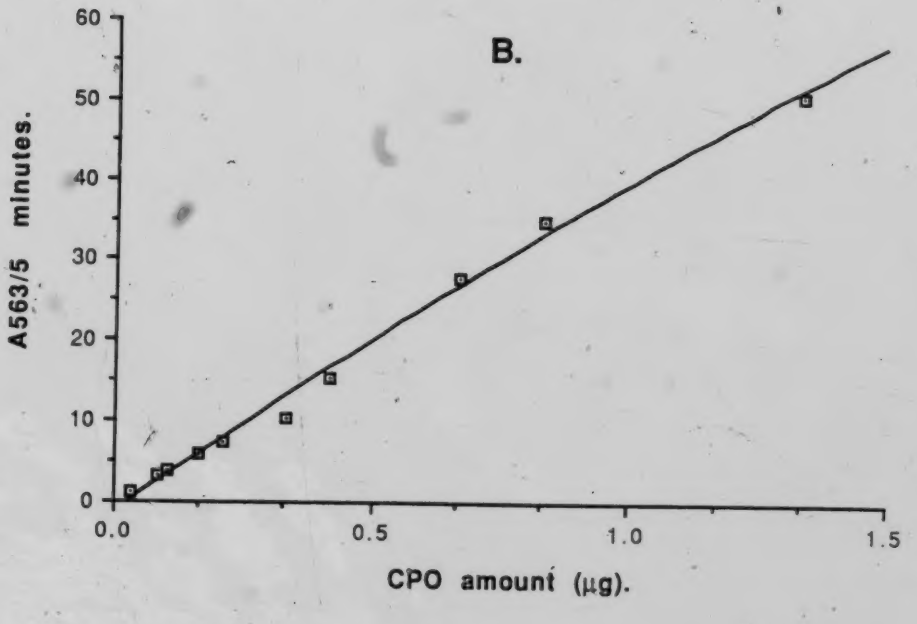
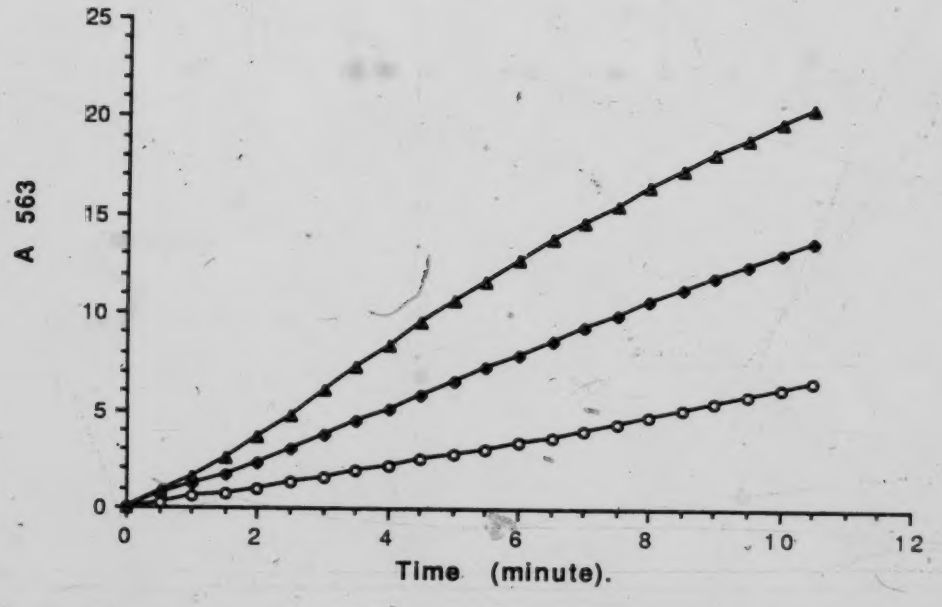

B.
A563/5 minutes.
60
50
40
30
20
10
0
0.0
0.5
1.0
1.5
CPO amount (µg).

Figure 3.7. Effect of reaction time on product formation during the chloroperoxidase assay.

The relationship was studied over a 12-minute period, at room temperature. Different amounts of CPO, 67 ng (o), 134 ng (♦), and 201 ng (Δ), were added to 1-ml reaction mixtures containing equal amounts of hydrogen peroxide (1 μmol), TMPD (20 μmol), and 100 μmol phosphate buffer, pH 6.0.


25
20
15
10
5
0
A 563
0
2
4
6
8
10
12
Time (minute).

CPO was in the treatment of industrial wastewaters containing phenolics, a suitable immobilization carrier for this system was glass or sand. Two important aspects of these inorganic supports were their inertness to microbial degradation and their mechanical strength (Haller 1983). Therefore, the choice of the immobilization method was limited to those applying to these carriers. In addition to the nature of the support, the procedure adopted for binding CPO to glass was dictated by the stability profiles of the soluble enzyme to temperature and pH, and by the nature of interaction responsible for immobilization. On the basis of the stability studies, the conditions for CPO immobilization were restricted to a pH range of 3.0 to 7.0, to avoid the acid denaturation at pH <2.8 and irreversible alkaline transitions at pH >7.0 (Lambeir and Dunford 1983b), and to a temperature below 45°C (Pickard and Hashimoto 1988). The binding method of choice was covalent, because it resulted in a stronger bonding of the enzyme to the carrier and produced a more stable immobilized enzyme derivative (Kennedy and Cabral 1987).

3.2.1 Glutaraldehyde linker method.

To covalently bind enzymes to glass beads, the support had to be first derivatized. The derivatization of glass beads was carried out following the methodology previously described by (Weetall 1976). This method is termed the glutaraldehyde linker method, because the enzyme was presumably immobilized to the carrier through a glutaraldehyde linker arm, which had been attached to the aminopropyl-glass in a distinctly seperate reaction step, as shown in Figure 2.1.

The glass beads were aminated in a solution of aminopropyltriethoxysilane (APES). However, since previous reports (Weetall 1976, Olsson and Ögren 1983) indicated that the type of the solvent used in the amination reaction affected the final enzyme loading capacity. Therefore, a study was conducted to determine whether the

organic silanization or the aqueous silanization procedure would load higher amount of amine groups onto the carrier. In this study, and in most of the initial CPO immobilization studies, a 40-mesh particle size glass was used. This carrier was easier to handle than the fine powder-like porous beads, because of its larger size. The results in Table 3.3 indicated that the aqueous silanization method gave higher CPO loading capacity than the acetone or the toluene silanization methods. The aqueous silanization of glass was therefore chosen as the standard amination procedure.

In the second and last steps, the aminated glass was treated with glutaraldehyde and then reacted with the enzyme. During the enzyme immobilization reaction step, 33.5 mg CPO were presented to 1 g of glass in 500 µl buffer solution. Despite this relatively high concentration of CPO per unit carrier, only a very small fraction of added enzyme, representing less than 0.1%, was bound to the glass. Because of this low coupling yield, the quantitation of the amount of CPO bound to the glass was based on the total activity of the immobilized enzyme derivative. The highest activity of the enzyme bound on 40-mesh glass corresponded to 6.3 µg of soluble CPO (Table 3.3). This loading capacity compared poorly to the 25 to 42 µg of alcohol dehydrogenase and L-lactate dehydrogenase immobilized by Brotherton *et al.* (1976), on 50-mesh glass, a carrier of only slightly different surface area. These results indicated that either CPO had a lower specific activity in the immobilized form or the immobilization procedure was not used under optimal conditions.

3.2.1.1 Optimization of the amination step.

To define the optimum conditions of the amination reaction, a number of physical and chemical parameters were analyzed. In every case, the complete sequence of reactions was carried out, with only one parameter varied, to determine the effect of the variable on the amount of CPO activity per unit mass of glass. These variables

Table 3.3. Effect of solvent on the amination of 40-mesh glass beads.

Solvent	immobilized CPO (μg / g of glass)(1)	Relative bound activity (%) mean(2)	(± S.D)
Acetone	3.1	49.0	9.6
Toluene	1.5	24.3	4.8
Water	6.3	100	8.0

(1) The amount of CPO immobilized was determined from the total activity of the immobilized enzyme derivative, and the specific activity of the soluble enzyme. The amination reaction was carried out in the three solvents following the procedure described in section 2.5.1.1. The aminopropyl-glass particles thus prepared were then treated with glutaraldehyde. CPO was bound to this material by the glutaraldehyde linker method and the bound activity determined in a 5-minute colorimetric assay. (2) Mean of 3 values.

included the silanization reaction temperature, time, pH, and the concentration an volume of the APES solution.

3.2.1.1 Optimization of the amination step.

To define the optimum conditions of the amination reaction, a number of physical and chemical parameters were analyzed. In every case, the complete sequence of reactions was carried out, with only one parameter varied, to determine the effect of the variable on the amount of CPO activity per unit mass of glass. These variables included the silanization reaction temperature, time, pH, and the concentration and volume of the APES solution.

By varying the temperature and time of the reaction, two profiles were obtained (Figure 3.8). The results showed that the amount of bound activity increased with both the reaction time and temperature, up to a maximum amount of bound activity. This maximum bound activity was obtained at 70°C, after a 3-hour reaction. Further increase in reaction temperature or reaction time resulted in a decrease of bound activity.

The study of the effect of APES concentration on CPO immobilization revealed an initial dependence of the amount of bound activity on APES concentration. The maximum loading capacity was achieved at 5% APES (Figure 3.9A). Further increase in APES concentration did not increase the binding of the enzyme to the glass. The shape of the curve indicated that the amination sites on the carrier were saturated. To ensure that APES would not become limiting during the amination of high surface area porous glass, a 10% APES concentration was selected for the amination reaction. The literature-recommended volume of the APES solution was 15 ml per 1 g of glass (Olsson and Ögren 1983, Weetall 1976). However, from this study, it was found that a 20 ml-solution gave slightly higher bound activity (Figure 3.9B). Therefore, per gram of glass, 20 ml of this solution was routinely used in subsequent amination reactions.

Figure 3.8. Dependence of CPO loading on the silanization reaction time and temperature.

The reaction mixture contained 1 g of 40-mesh glass and 15 ml of a 10% aqueous APES solution, pH 3.45. **A.** The effect of the reaction time was determined by carrying out the silanization of a set duplicate glass bead samples at a constant temperature (75°C), for different time periods. **B.** The effect of the reaction temperature was determined by carrying out the silanization of a set of duplicate glass bead samples for 3 hours, at different temperatures.

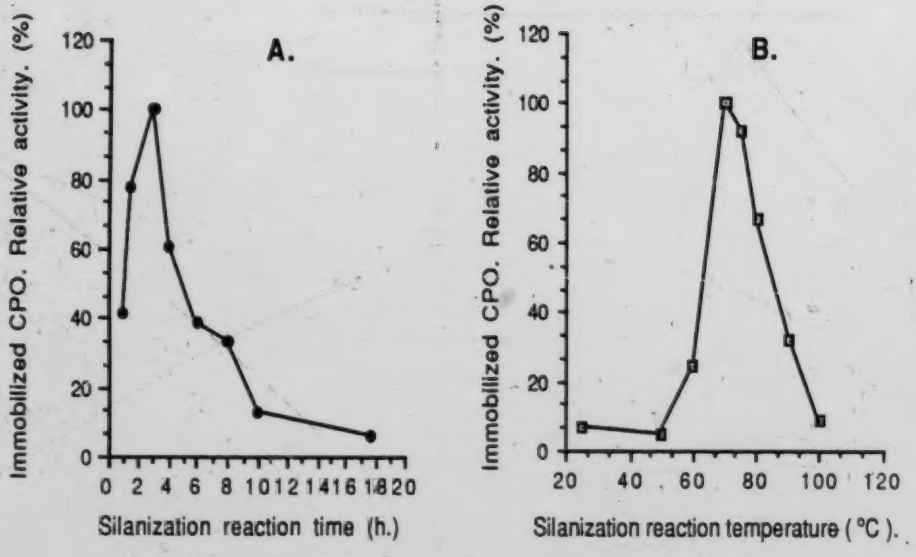

A.
Immobilized CPO. Relative activity. (%)
120
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20
Silanization reaction time (h.)
B.
Immobilized CPO. Relative activity. (%)
120
100
80
60
40
20
0
20 40 60 80 100 120
Silanization reaction temperature (°C).

Figure 3.9. Dependence of CPO loading on the APES concentration and volume.

A. The effect of APES concentration was determined by carrying out the silanization of a set of duplicate 1-g samples of 40-mesh glass beads at different concentrations of APES. The silane solutions were prepared in milli Q water, and the pH was adjusted to 3.45 with concentrated HCl. **B**. The effect of volume of 10% APES solution, pH 3.45, was determined by carrying out the silanization of a set of duplicate 1-g samples of 40-mesh glass beads in different volumes of APES solution. The reactions were carried out at 75°C, for 3 hours.

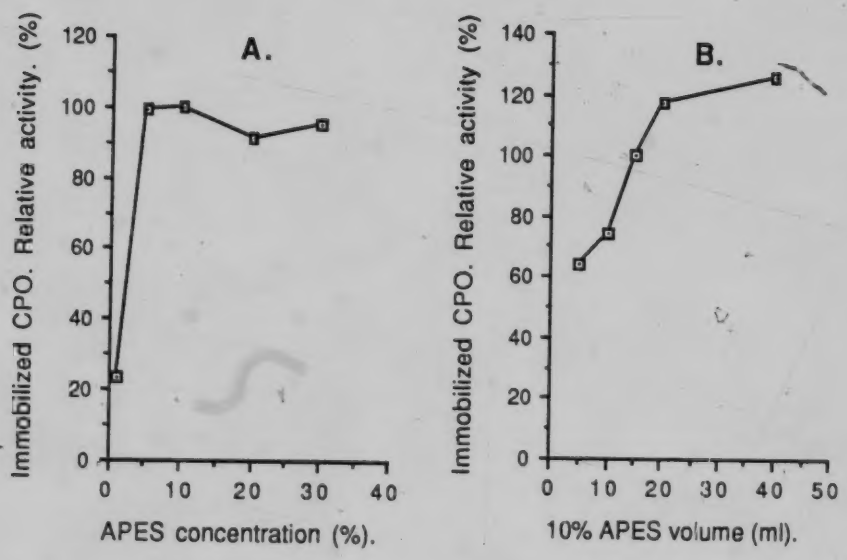

A.
Immobilized CPO. Relative activity. (%)
120
100
80
60
40
20
0
0
10
20
30
40
APES concentration (%).
B.
Immobilized CPO. Relative activity (%)
140
120
100
80
60
40
20
0
0
10
20
30
40
50
10% APES volume (ml).

Table 3.4. Effect of the silanization reaction pH on the immobilization of CPO on 40-mesh glass beads.

pH	Bound CPO (μg/g glass)(1)	Relative bound activity (%)
2.0	15.0	89.1
3.0	18.3	108
4.0	16.8	100
5.0	18.8	112
6.0	15.8	93.7
7.0	21.2	126
8.0	15.8	93.7
9.0	17.4	103
10.0	19.1	113
10.9	15.8	93.6

(1) The amount of CPO immobilized was determined from the total activity of the immobilized enzyme derivative, and the specific activity of the soluble enzyme. The amination reaction was carried out at different pH values in 10% aqueous APES solution (15 ml), for 3 hours, at 70°C. The aminopropyl-glass particles thus prepared were then treated with glutaraldehyde. CPO was bound to this material by the glutaraldehyde linker method and the bound activity determined in a 5-minute colorimetric assay.

The pH of the APES solution did not seem to have any effect on the amount of immobilized CPO (Table 3.4). In keeping with the literature procedure, a pH of 3.45 was chosen.

In summary, the optimum conditions for the glass amination reaction were determined to be: 20 ml of 10% aqueous APES solution, pH 3.45 were added to 1 g of glass beads, and the reaction was allowed to proceed for 3 hours, at 70°C.

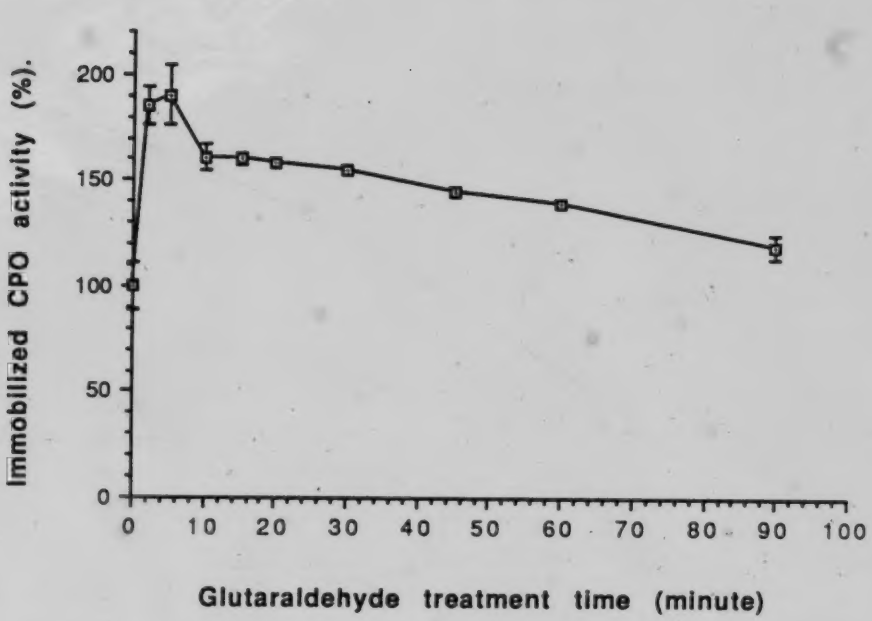
3.2.1.2 Optimization of the glutaraldehyde step.

The second immobilization step involved the treatment of the aminopropyl-glass with glutaraldehyde. As in the previous immobilization step, the effects of the different glutaraldehyde reaction parameters on CPO immobilization were investigated. The effects of (i) the glutaraldehyde concentration, (ii) the buffer concentration, and (iii) the reaction pH were studied. The results from these studies confirmed the reaction conditions described in literature (section 2.5.1.2). However, an important observation made during the study of the reaction time indicated that a shorter treatment of the alkylamine glass with glutaraldehyde resulted in higher levels of bound activity than those obtained with a longer treatment (Figure 3.10). The maximum bound activity was achieved within 2 to 5 minutes, and a 90-minute glutaraldehyde treatment resulted in a 50% decrease from the maximum bound activity. It was also observed that a significant amount of activity, about 50% of the maximum, was bound to the glass in the absence of the glutaraldehyde treatment. Further investigation of this observation lead to the development of the modified immobilization procedure discussed in section 3.2.2 below.

In summary, the optimum conditions of the glutaraldehyde treatment of the aminated glass were as follows: 5 ml of 2.5% glutaraldehyde solution in 100 mM phosphate buffer, pH 7.0 were added to 1 g of aminated glass, and the reaction was

Figure 3.10. Effect of glutaraldehyde treatment time on CPO immobilization on glass beads.

The effect of glutaraldehyde treatment time was determined by adding 2.5% glutaraldehyde (5 ml), prepared in 100 mM phosphate buffer, pH 7.0, to a set of 0.1-g samples of 40-mesh aminopropyl-glass beads.The reaction was carried out at room temperature, with occasional shaking of the samples. After different times, the reaction was stopped by washing the samples extensively with glass-distilled water, prior to the addition of CPO (300-400 μg) to this material. The enzyme immobilization reaction was carried out at room temperature, in 1 ml 100 mM phosphate buffer, pH 5.5, overnight. The amount of bound activity was determined in a 5-minute colorimetric assay, after the immobilized enzyme material was extensively washed with 250 mM phosphate buffer, pH 5.5. Points displayed are means ± SD from three determinations.


Immobilized CPO activity (%).
200
150
100
50
0
0
10
20
30
40
50
60
70
80
90
100
Glutaraldehyde treatment time (minute)

carried out for 5 to 10 minutes, at room temperature.

3.2.1.3 Optimization of CPO immobilization step.

To determine the optimum conditions for the immobilization reaction step, the effects of (i) the reaction temperature, (ii) the enzyme and (iii) buffer concentrations on CPO immobilization were studied. To analyze the effect of the temperature on CPO immobilization, the reaction was carried at three different temperatures, 5, 15 and 22°C, and the amount of bound activity was determined. The loading of CPO on glass increased with the immobilization reaction temperature (Table 3.5). This suggested that by increasing the temperature the enzyme likely adopted a conformation which exposed the chemical groups involved in the binding to the glass, or the bonding reaction between the enzyme and the derivatized carrier was presumably favored at room temperature (the upper temperature tested).

The effect of enzyme amount presented to the carrier was also analyzed. In this study, different amounts of enzyme were added to aminopropyl-glass. Two concentrations of the phosphate buffer, 20 and 100 mM, were used to also determine the effect of the buffer concentration on CPO immobilization. The results showed that the coupling of CPO to glutaraldehyde pretreated 40-mesh alkylamine glass was affected by both the enzyme and buffer concentrations (Figure 3.11). At 20 mM phosphate buffer concentration, the enzyme immobilization profile indicated an initial increase in bound activity with increase in the amount of enzyme presented to the carrier. In comparison to the activity of the soluble enzyme, the maximum bound activity determined corresponded to 25 to 30 μg of immobilized CPO at 200-400 μg of enzyme presented to the glass. Subsequent increase of the amount of CPO presented to the carrier resulted in a decrease of bound activity. The minimum reaction volume was not tested in this study. At 100 mM phosphate buffer, the same profile was obtained.

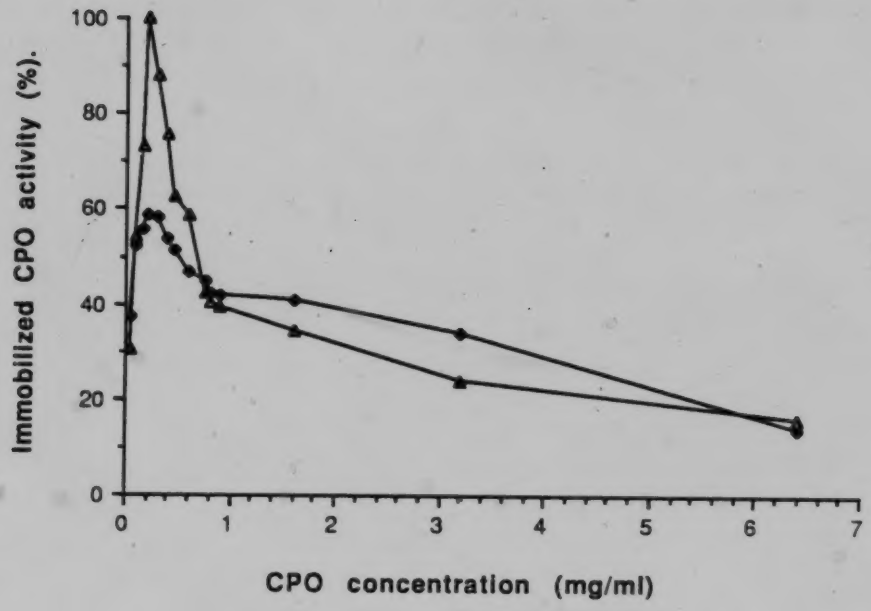
Table 3.5. Effect of immobilization reaction temperature on the binding of CPO on derivatized glass beads.

Temperature (°C)	Bound CPO (μg/g glass)(1)	Relative bound activity (%)
5	6.5	100
15	8.6	133
22	12.0	184

(1) The amount of CPO immobilized was determined from the total activity of the immobilized enzyme derivative, and the specific activity of the soluble enzyme. The amination reaction was carried out at with 40-mesh glass (1 g) in 10% aqueous APES solution, pH 3.45 (20 ml), for 3 hours, at 70°C. The aminopropyl-glass particles thus prepared were then treated with glutaraldehyde. CPO was bound to this material by the glutaraldehyde linker method at different temperatures, and the bound activity was determined in a 5-minute colorimetric assay.

Figure 3.11. Effects of buffer and enzyme concentrations on CPO immobilization on glass beads.

The effects of the buffer and enzyme concentrations on the immobilization of CPO on derivatized glass beads were determined by adding different amounts of enzyme (0.05-6.7 mg CPO) to 0.1-g samples of glutaraldehyde-treated 40-mesh aminopropyl-glass beads. The reaction was carried out at room temperature, overnight, in 1 ml of 20 mM (Δ) and 100 mM (♦) phosphate buffers, pH 5.5, with occasional shaking of the samples. The amount of bound activity was determined in a 5-minute colorimetric assay, after the immobilized enzyme material was extensively washed with 250 mM phosphate buffer, pH 5.5. Points displayed are means from two determinations.


Immobilized CPO activity (%).
100
80
60
40
20
0
0
1
2
3
4
5
6
7
CPO concentration (mg/ml)

However, the bound activity was lower than that obtained at 20 mM. During these studies, it was also observed that CPO could not bind to non-aminated acid-washed glass beads.

In summary, the optimum conditions of the CPO immobilization reaction step were as follows: to 1 ml of 200 to 400 mg of CPO in 20 mM phosphate buffer, pH 5.5, was added 1 g of glass, and the reaction was carried out overnight at room temperature. The effect of the reaction time on CPO immobilization was not determined in this study. However, a study of the immobilization reaction time was carried out in another experiment using controlled-pore glass beads (section 3.2.2.1 below).

3.2.2 Glutaraldehyde crosslinking method.

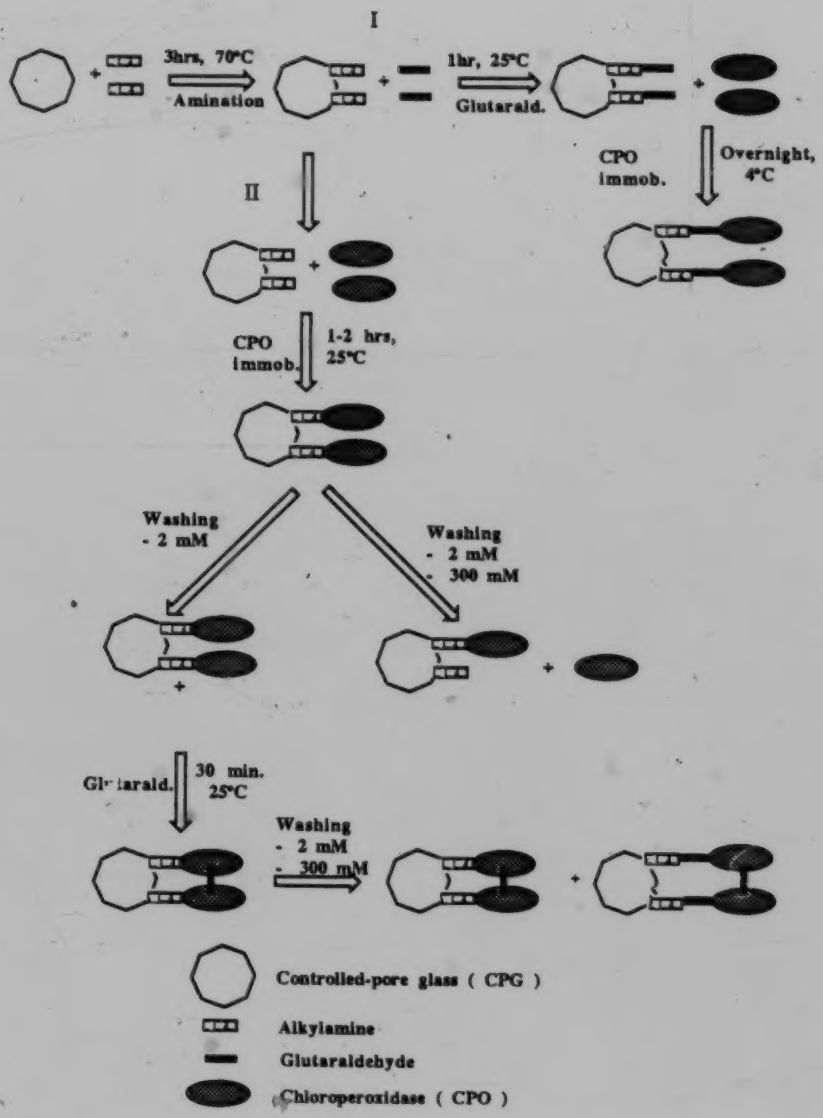
The observations made during the optimization of the glutaraldehyde reaction and the enzyme reaction steps above, indicated that (i) CPO could be attached to aminated glass in the absence of glutaraldehyde; (ii) increasing the concentration of CPO presented to the glass gave rise to bound activity profiles with a bell shaped curve rather than the expected curve with a plateau, which would have indicated saturation of the carrier; and (iii) decreasing the ionic strength of the CPO immobilization reaction mixture increased the amount of bound activity, implying ionic rather than covalent bonding.

On the basis of these observations, some changes were made to the immobilization method described in section 3.2.1. This modification is schematically represented in Figure 3.12. As with the previous methodology, there are still three major steps: (i) the amination of the glass, (ii) the CPO immobilization reaction, and (iii) the glutaraldehyde crosslinking reaction, but the order has changed.

To investigate the mechanism involved in CPO binding to derivatized glass, an experiment was carried out in which CPO was directly reacted with alkylamine glass in

Figure 3.12. The scheme of CPO immobilization on glass beads by the published and modified methods.

The scheme shows the published glutaraldehyde linker method in **pathway I**, and the modified method, the glutaraldehyde crosslinking method in **pathway II**. In the glutaraldehyde crosslinking method, the enzyme immobilization reaction step precedes the glutaraldehyde treatment step. 2 and 300 mM refer to the concentrations of the washing buffer, potassium phosphate, pH 4.0. The final immobilized enzyme preparation presumably consists of two products resulting from: (i) CPO directly bound to aminopropyl-glass and (ii) CPO bound to aminopropyl-glass via a glutaraldehyde linker arm as in pathway I. In both binding types, adjacent enzymes are crosslinked with glutaraldehyde.


I
3hrs, 70°C
Amination
1hr, 25°C
Glutarald.
CPO
immob.
Overnight,
4°C
II
CPO
immob.
1-2 hrs,
25°C
Washing
- 2 mM
Washing
- 2 mM
- 300 mM
Glutarald.
30 min.
25°C
Washing
- 2 mM
- 300 mM
Controlled-pore glass (CPG)
Alkylamine
Glutaraldehyde
Chloroperoxidase (CPO)

a buffer of low ionic strength. In this study, alkylamine controlled-pore glass, CPG-75, was used as the immobilization support. The immobilization reaction was carried out at room temperature with occasional mixing. The immobilized enzyme was subjected to different washing conditions, then treated with glutaraldehyde. It was observed that, within a few minutes of the initiation of the immobilization reaction, CPO was rapidly removed from the solution. The color of the aminopropyl-glass changed from whitish to brownish red, as the carrier picked up the enzyme from solution (Figure 3.13). Washing the immobilized CPO derivative with high ionic strength buffer resulted in the release of a large portion of the enzyme from the alkylamine glass. This implied that the binding of CPO to alkylamine glass was ionic, not covalent.

The rapid concentration of CPO on the aminated carrier indicated that strong attraction existed between CPO and the carrier under the conditions of the experiment. And this attraction likely resulted from electrostatic forces between the enzyme and the aminated carrier. However, when the immobilized CPO derivative was washed with low ionic strength buffer and subsequently treated with glutaraldehyde before the final washing with high ionic strength buffer, higher retention of the bound enzyme was observed. Presumably the glutaraldehyde treatment crosslinked the bound enzyme and minimized the release in the presence of high ionic strength buffer. The results of this survey experiment on the effect of the different washing conditions are presented in Table 3.6. Since the direct immobilization of CPO on alkylamine glass was strongly affected by the ionic strength and possibly by the pH of the reaction mixture as well, the second step of the modified methodology - the enzyme immobilization reaction - was termed the ionic binding step. A comparison of the loading capacity achieved with the two methods of CPO immobilization - the glutaraldehyde linker method and the glutaraldehyde crosslinking method - indicated that the latter resulted in a two-fold

Figure 3.13. CPO immobilized on controlled-pore glass.

Shown are the vials from a survey experiment containing 20 mg of non-aminated or aminated 200-400-mesh controlled-pore glass beads (75 Å pore size), to which 1 ml of CPO (6 mg/ml) in 20 mM phosphate buffer, pH 5.5, was added. The enzyme immobilization reaction was carried out initially under reduced pressure (15 to 20 minutes) and then at room temperature, for 2 hours. The immobilized enzyme material was washed in 20 mM phosphate buffer, pH 5.5, then treated or not treated with 1 ml of 2.5% glutaraldehyde in 50 mM phosphate buffer, pH 5.5, and finally subjected to a variety of washing conditions. The vials contained from left to right: (**A**) control: the non-aminated glass reacted with the enzyme, then washed; (**B**) the immobilized enzyme not crosslinked but washed; (**C**) the immobilized enzyme not crosslinked and not washed; (**D**) the immobilized enzyme crosslinked but not washed; and (**E**) the immobilized enzyme crosslinked and then washed. Crosslinking refers to the glutaraldehyde treatment, and washing refers to the extensive washing of the material with 100 mM phosphate buffer, pH 5.5. When the washing was omitted the the immobilized enzyme was stored in 20 mM of the same buffer.

A. B. C. D. E.

Table 3.6. Effects of glutaraldehyde crosslinking and washing buffer concentration on CPO immobilized on aminopropyl-controlled-pore glass.

Immobilization step.	washing buffer concentration		glutaraldehyde crosslinking	washing buffer concentration		Immobilized CPO (%)
	20 mM	100 mM		20 mM	100 mM	
CPO immobilization step.	+	-	-	-	-	92.8
	+	+	-	-	-	33.2
Glutaraldehyde crosslinking step.						
	+	-	+	+	-	100
	+	-	+	+	+	76.9
CPO immobilization by the G.L. method (1)	+	+	NA (2)	NA	NA	49.8

(1). The CPO immobilization step of the glutaraldehyde linker method is included for comparison to the glutaraldehyde crosslinking method. (2). NA: not applicable to this immobilization method. The table shows the results from the survey experiment described in Figure 3.13. The immobilized enzyme material was washed with the buffer of different concentrations, and at various stages of the enzyme immobilization by the glutaraldehyde crosslinking method. The amount of bound protein was then determined by the "difference method" and the modified Lowry assay. The amount of bound protein shown in the last column was an average of two measurements from the two protein assays. The difference in the measurements by the two methods was less than 10%.

increase of the enzyme loading (Table 3.6). Subsequent studies were conducted to optimize the immobilization reaction step of the modified method.

3.2.2.1 Optimization of the CPO immobilization step.

Further studies were carried out to define the optimum conditions for the immobilization of CPO by the crosslinking method. The immobilization reaction was conducted with occasional mixing of the reaction mixture at different times, and the amount of CPO immobilized was quantitated by the difference of CPO remaining in solution and the amount of CPO presented to the carrier. Analysis of the effect of the enzyme immobilization reaction time on ionic binding of CPO to alkylamine glass indicated that about 80% of the reaction was completed in about 1 hour (Figure 3.14). The immobilization reaction was considered to be approaching completion, when the rate of the binding of the enzyme to the carrier became negligible, and the enzyme loading reached a plateau. The buffer concentration and pH affected the loading capacity: increasing the buffer concentration resulted in a proportional decrease in the amount of CPO immobilized (Figure 3.15A); increasing the pH resulted in an increase in loading capacity, up to a maximum level obtained at pH 6.0, and this increase was followed by a slight decrease beyond pH 6.0 (Figure 3.15B). Because pH 7.0 was the highest pH tested, it was assumed that the observed decrease in loading capacity beyond pH 6.0 indicated a trend. It is likely that the amount of immobilized CPO continued to decrease with increasing pH. At pH 3.0 and below, CPO did not bind to the aminated glass. This results indicated that the overall charge of the enzyme influenced the outcome of the immobilization reaction. On the basis of the enzyme isoelectric point (pI 3.6-3.9) (Sae and Cunnigham 1979, Pickard and Hashimoto 1982), at pH 3.0 CPO had a net positive charge which precluded interaction with the cationic alkylamine glass. The binding of the enzyme to non-aminated acid-washed

Figure 3.14. Effect of immobilization reaction time on the ionic binding of CPO on aminopropyl-glass beads.

Aminopropyl-CPG-75 (20 mg) was added to CPO (7mg) in 1 ml of 2 mM phosphate buffer, pH 6.0. The reaction was carried out at room temperature with occasional shaking. The immobilized enzyme was washed with 2 mM buffer, pH 5.5, and the amount of bound protein was determined from the washings by the "difference method".

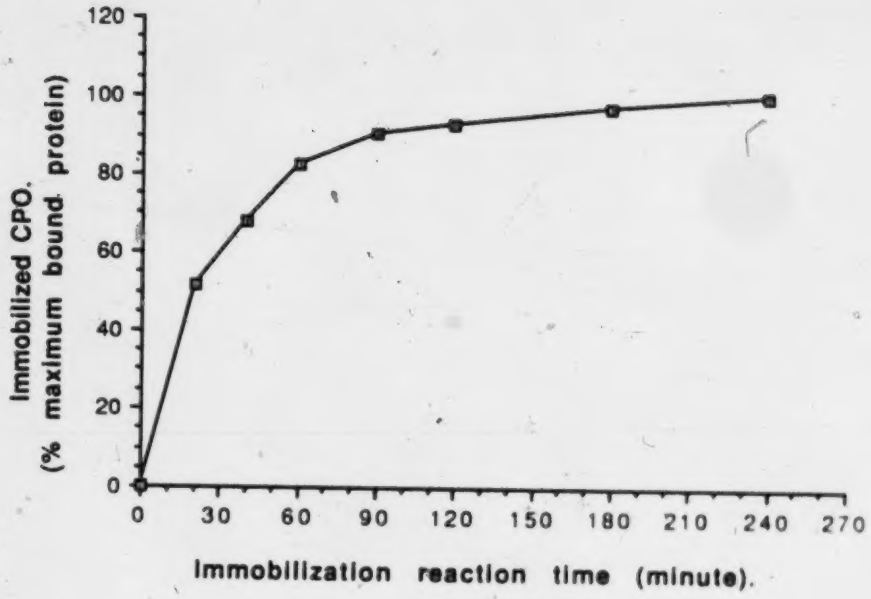
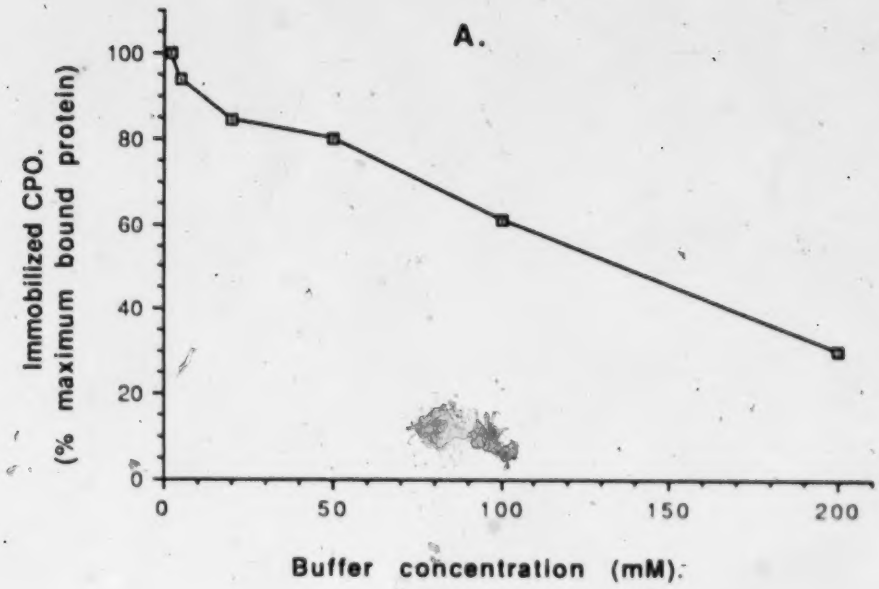
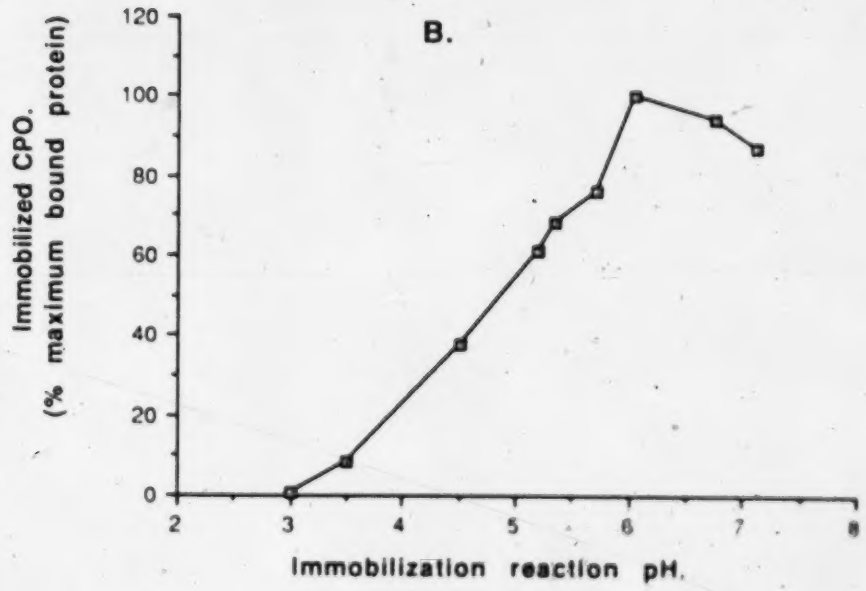

Immobilized CPO.
(% maximum bound protein)
120
100
80
60
40
20
0
0
30
60
90
120
150
180
210
240
270
Immobilization reaction time (minute).

Figure 3.15. Effects of immobilization reaction pH and buffer concentration on the ionic binding of CPO on aminopropyl-glass beads.

Aminopropyl-CPG-75 (20 mg) was added to CPO (7 mg) in 1 ml of 2 mM phosphate buffer, pH 6.0. **A.** The reaction was carried out at room temperature in buffers of different concentrations, at pH 6.0. **B.** The reaction was carried out at room temperature with occasional shaking, in 2 mM phosphate buffer, at different pH values. The immobilized enzyme was washed with 2 mM buffer, pH 5.5, and the amount of bound protein was determined from the washings by the "difference method".


A.
Immobilized CPO.
(% maximum bound protein)
100
80
60
40
20
0
0
50
100
150
200
Buffer concentration (mM).


B.
Immobilized CPO.
(% maximum bound protein)
120
100
80
60
40
20
0
2
3
4
5
6
7
8
Immobilization reaction pH.

porous glass was negligible.

In summary, the immobilization of CPO was optimally carried out in a low ionic strength buffer, at pH 6.0. The reaction was allowed to proceed at room temperature for a maximum of 2 hours, with occasional mixing.

3.2.2.1.1 Effect of enzyme:carrier ratio on CPO immobilization.

The results on the effect of enzyme loading indicated an apparent saturation of the carrier at 8 mg CPO/g glass (Figure 3.16, and Table 3.7). A similar apparent saturation of the carrier, without a decreased activity at high loadings, has been recently reported during the immobilization of glucose isomerase on porous SiO_2 carrier material by the glutaraldehyde linker method (Sørensen and Emborg 1989). The increase in amount of bound protein with the concentration of CPO in the immobilization reaction mixture, presumably resulted from the stacking of the enzyme on the alkylamine glass. If this is the case, a higher specific activity would be expected if the concentration of the enzyme presented to the carrier was decreased. Therefore, the effect of the amount of CPO presented to the carrier was studied. An almost linear increase in the amount of bound protein was observed up to 100 mg CPO/g controlled-pore glass. However, at high bound protein levels, the specific activity of the enzyme was lowest. The data in Table 3.7 indicated that by decreasing the amount of CPO in the immobilization reaction mixture from 202 to 12.7 mg/ml, a 4.5 fold increase in specific activity was achieved.

3.2.2.1.2 Effect of carrier pore size on CPO immobilization.

The poor relationship between the immobilized enzyme activity and the amount of bound protein could be in part explained on the basis of carrier pore size. If the activity detected was that expressed by the enzyme on the surface of the carrier, and that

Figure 3.16. Effect of enzyme concentration on the loading of CPO on aminopropyl-glass particles.

Different amounts of CPO were presented to 20-mg samples of aminopropyl-CPG-240 in 2 mM phosphate buffer, pH 6.0. The ionically bound enzyme preparations were subsequently washed with 2 mM buffer, pH 6.0, then treated with 0.5 ml 2.5% glutaraldehyde in 5 mM buffer, pH 6.0). **Covalent step (protein)**: the immobilized enzyme materials were finally washed with phosphate buffer of high ionic strength (500 mM, pH 5.5). The amount of protein and activity of the enzyme covalently bound on the carrier were determined by the modified Lowry and the standard chloroperoxidase colorimetric assay, respectively. **Covalent step (activity)**: based on the specific activity of soluble CPO, the amount of active CPO was calculated. The specific activities were calculated from these two sets of values and are shown in Table 3.7 as bound CPO specific activity.

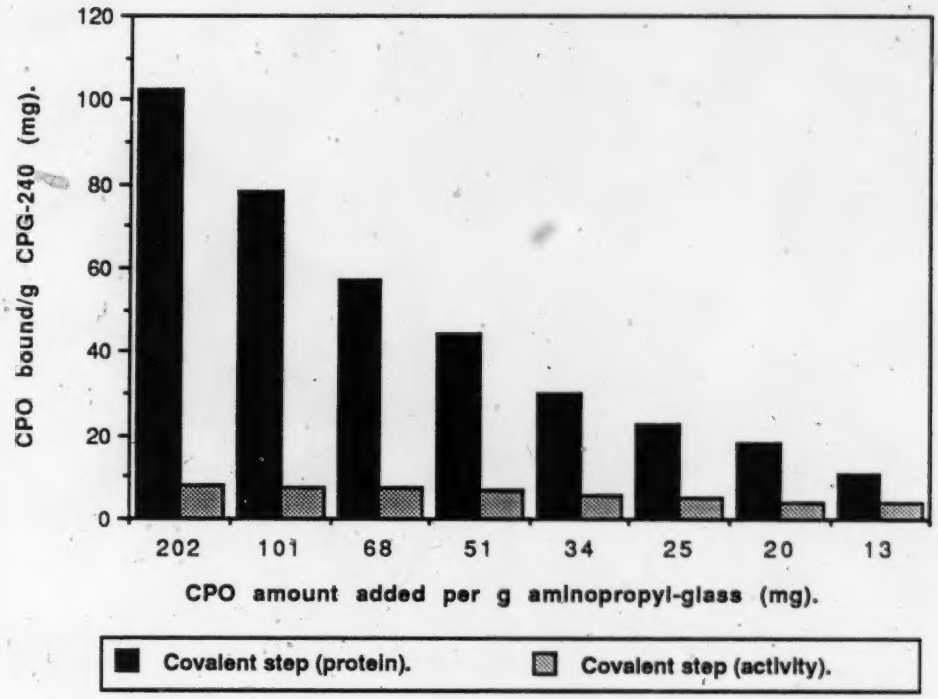

120
100
80
60
40
20
0
CPO bound/g CPG-240 (mg).
202
101
68
51
34
25
20
13
CPO amount added per g aminopropyl-glass (mg).
Covalent step (protein).
Covalent step (activity).

Table 3.7. Effect of enzyme amount presented to porous aminopropyl-CPG-240 on the specific activity of immobilized CPO.

CPO presented to CPG-240 (mg/g glass)	CPO (protein) covalently bound (mg/g glass)	Active CPO covalently bound (mg/g glass)	Bound CPO specific activity (%)[(1)]
202	102	8.1	7.9
101	78.3	7.1	9.1
67.5	57.1	7.6	13.3
50.6	44.0	6.9	15.7
33.7	29.9	5.9	19.7
25.3	22.8	4.9	21.5
20.2	18.3	3.9	21.3
12.7	10.7	3.9	36.4

(1). The specific activities were calculated as described in the legend to Figure 3.16. This Table shows the data from Figure 3.16, for clarity on the effect of the amount of CPO presented to the carrier on the coupling yield.

the large surface area of the porous glass (Filbert 1975, Haller 1983) was not being efficiently utilized, a higher specific activity of the immobilized enzyme would be expected with increase in the pore size (Weetall 1975). The increase in the pore size is expected to achieve high specific activity only when the pores are of the right size to allow both the enzyme and its substrates to enter.

To get an indication of the effect of pore size on enzyme loading, CPO was immobilized on porous glass beads having the same bead size (200- to 400-mesh), but different pore sizes (75, 170, 240, and 540 Å). The preparations were compared by the relative amount of enzyme bound during the ionic step. The results indicated that the highest enzyme loading was achieved at using 170 Å pore size (Table 3.8). The specific activity determined after the covalent step was also highest with CPG-170. A 60% reduction in the amount of bound enzyme occurred when CPO was immobilized to alkylamine glass with 540-Å pores. This decrease in the amount of enzyme immobilized from 170- to 540-Å pore sizes was directly related to the decrease in the surface area resulting from the increase in the pore size beyond a critical size. This critical pore size appeared to be around 170 Å for CPO. Despite the relative increase in the amount of enzyme immobilized on CPG-170, no significant increase in the overall specific activity was observed with any of the four immobilized enzyme preparations. These results suggested that the low specific activity of immobilized CPO was not due to lack of enzyme access to the carrier pores, but presumably to "over-crowding" of the carrier. Alternatively, the reduced specific activity may be an inherent characteristic of the immobilized enzyme.

3.2.2.2 Optimization of the glutaraldehyde crosslinking step.

The effect of glutaraldehyde concentration during the crosslinking reaction step was also investigated. The results indicated no effect of glutaraldehyde on the enzyme

Table 3.8. Effect of pore size on the ionic binding of CPO to aminopropyl-controlled-pore glass.

Carrier-pore size (200-400-mesh size)	Typical specific surface area (m^2/g) (1)	Bound protein (mg/g glass) mean	(± S.D)
CPG-75	185	116	3.9
CPG-170	140	131	1.7
CPG-240	100	114	3.6
CPG-540	50	63.7	3.9

(1) The estimated surface areas from reviews by Haller (1983] and Filbert (1975). CPO was ionically bound to a set of aminopropyl-glass (20 mg samples) of different pore sizes, in 2 mM phosphate buffer, pH 6.0. The ionically bound enzyme preparations were subsequently washed with 2 mM buffer, pH 6.0, then treated with 0.5 ml 2.5% glutaraldehyde in 5 mM buffer, pH 6.0). The immobilized enzyme materials were washed with phosphate buffer of high ionic strength (500 mM, pH 5.5). The amount of protein covalently bound on the carrier was determined by the modified Lowry assay.

previously bound to alkylamine glass during the "ionic step" (Figure 3.17). However, increasing the concentration of glutaraldehyde beyond 0.62%, caused the release of a fraction of CPO previously bound to the carrier. The amount of enzyme released increased with glutaraldehyde concentration. In the subsequent washing of the immobilized enzyme material with high ionic strength buffer, another fraction of immobilized enzyme was released from the carrier. This washing step was performed to determine the amount of CPO covalently bound to the glass. When the overall amount of enzyme released (as determined from the last step) was correlated to the concentration of glutaraldehyde used to crosslink the enzyme, the results indicated that increasing the glutaraldehyde concentration, from about 0.6 to 2.5, did not have any effect on the amount of enzyme bound. In the same Figure, the activity of the immobilized CPO derivative treated with different glutaraldehyde concentrations was determined (Figure 3.17). A nearly constant amount of bound activity was obtained, corresponding to 8 mg of soluble CPO/g glass, and this was irrespective of the glutaraldehyde concentration used in step 2.

3.3 DETERMINATION OF CHARACTERISTICS OF SOLUBLE AND IMMOBILIZED CPO.

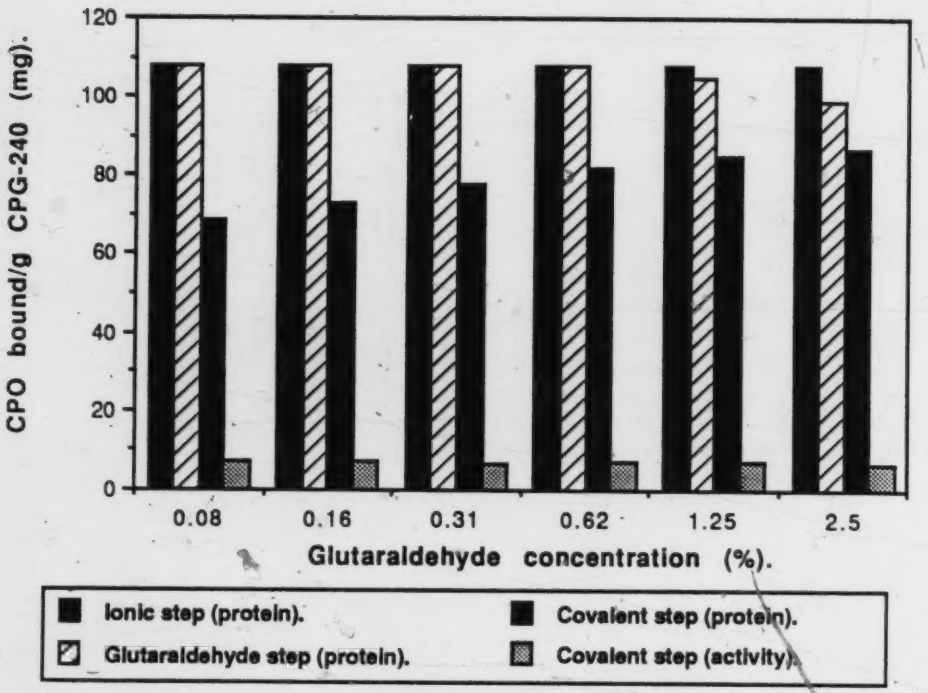
To determine the conditions for maximum stability and catalytic activity of chloroperoxidase and ensure efficient use of the immobilized enzyme, pH activity profiles, and stability under storage and operational conditions of CPO were studied.

3.3.1 pH activity optima of chloroperoxidase.

The effects of pH and reactants on the catalytic velocity of CPO were investigated during the oxidation of TMPD and phenol. The TMPD oxidation system

Figure 3.17. Effect of glutaraldehyde concentration on the stabilization of CPO ionically bound to aminopropyl-CPG-240, at different stages of the modified immobilization methodology.

CPO was ionically bound to a set of aminopropyl-glass (20 mg samples) in 2 mM phosphate buffer, pH 6.0. **Ionic step (protein)**: the amount of protein ionically bound to aminopropyl-glass in this step was determined by the "difference method". **Glutaraldehyde step (protein)**: the ionically bound enzyme material were subsequently washed with 2 mM buffer, pH 6.0, then treated with different conentrations of glutaraldehyde (prepared in 0.5 ml 5 mM buffer, pH 6.0) and the amount of enzyme remaining bound was again determined by the "difference method". **Covalent step (protein)**: to determine the amount of enzyme protein covalently bound to the carrier the immobilized enzyme materials were washed with phosphate buffer of high ionic strength (500 mM, pH 5.5). **Covalent step (activity)**: the activity expressed by the enzyme covalently bound on the carrier was determined by the standard colorimetric CPO assay and, based on the specific activity of soluble CPO, the amount of active CPO was calculated.


CPO bound/g CPG-240 (mg).
120
100
80
60
40
20
0
0.08
0.16
0.31
0.62
1.25
2.5
Glutaraldehyde concentration (%).
Ionic step (protein).
Glutaraldehyde step (protein).
Covalent step (protein).
Covalent step (activity).

was used for both the soluble and immobilized enzyme studies, whereas the phenol oxidation system was only used for the soluble enzyme studies.

3.3.1.1 pH activity profiles of TMPD oxidation.

The activities of soluble and immobilized CPO were determined at different pH values ranging from 2.75 to 7.0. During the course of the reaction, the pH of the reaction mixture did not change. The only change in reaction mixture pH occurred during the addition of TMPD, due to the acidity (pH 3.0) of the stock TMPD-dihydrochloride solution. The pH activity profiles of both systems indicated two pH optima, one near pH 2.8 and the other near pH 6.0. The curves of soluble and immobilized CPO were almost superimposable (Figure 3.18A). A slight increase in the relative specific activity of the immobilized CPO was also observed at the neutral pH optimum. Both soluble and immobilized enzyme expressed higher activity at the acidic pH optimum.

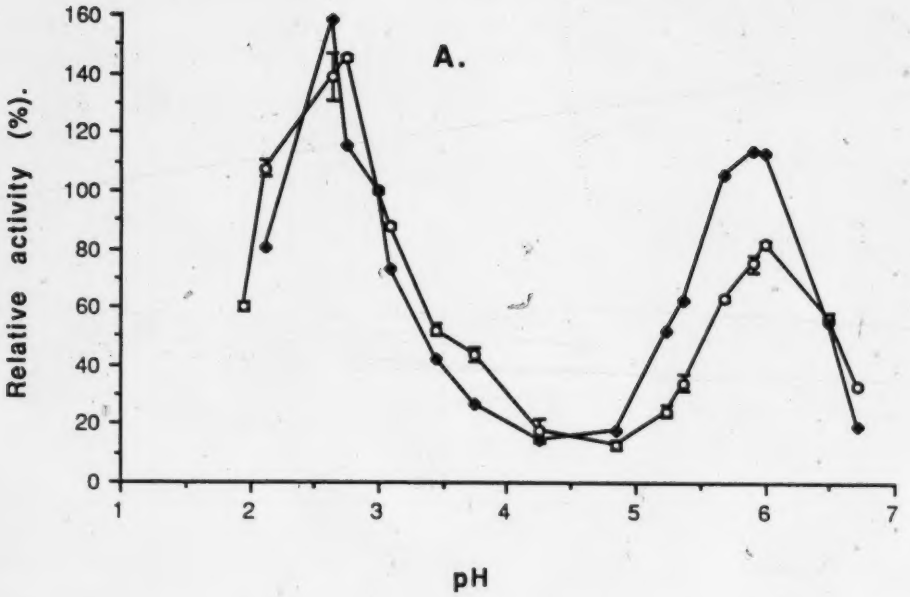
The two peaks presumably corresponded to the halide-dependent and halide-independent oxidation activities of CPO. The peak at pH 6.0 has previously been reported (Kedderis *et al.* 1980), and corresponded to the halide-independent demethylation activity of CPO. The peak at pH 3.0 has also been reported (Thomas *et al.* 1970), and corresponded to the halide-dependent demethylation activity (Kedderis *et al.* 1980). The two-peak pH activity profile observed in the present study, resulted from the presence of chloride ion in the reaction mixture. This anion derived from the salt form of the substrate compound - TMPD(dihydrochloride), used to overcome the limited solubility of TMPD in aqueous solutions.

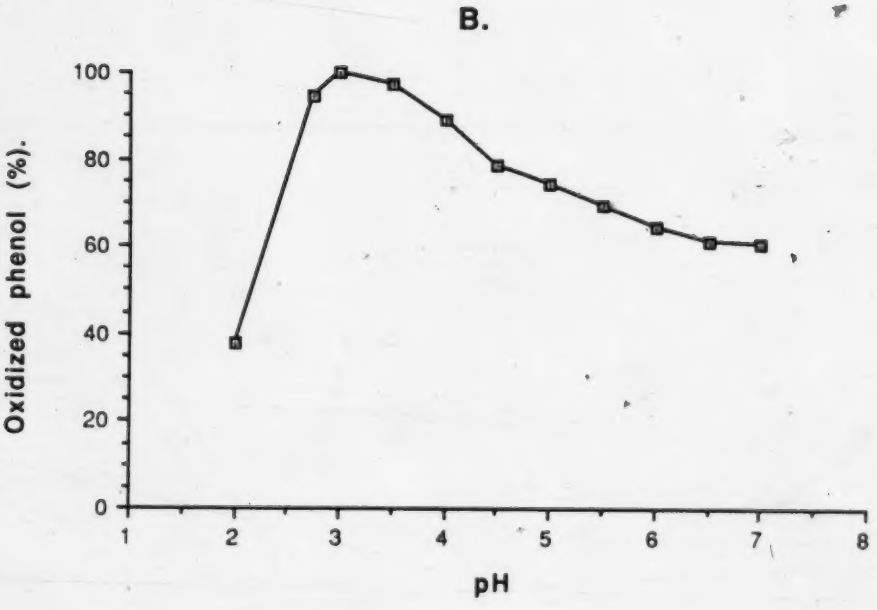
3.3.1.2 pH activity profile of phenol oxidation.

The effect of pH on CPO was also studied using a 2-minute phenol oxidation

Figure 3.18. pH activity profiles of CPO.

A. The pH activity profiles of soluble (o) and immobilzed (♦) CPO during TMPD oxidation were determined by measuring the rate of TMPD oxidation in the standard CPO colorimetric assay, at different pH values (from 2.0 to 6.7).The reaction mixtures for the soluble enzyme contained TMPD (20 μmol), hydrogen peroxide (1 μmol), CPO (0.2 μg) in 1 ml 100 mM buffer. Potassium phosphate buffer was used in the pH range of 5.0 to 6.7, and a mixture of this buffer with 100 mM phosphoric acid was used for pH values below 5.0. The pH was checked before and after the reaction, and no change was observed. **B.** The pH activity profile of soluble CPO during phenol oxidation was determined from phenol remaining in solution after a 2-minute reaction, carried at different pH values (2.75-7.0), was stopped by immersion in a 70°C water bath. The reaction mixture contained phenol (1 μmol), hydrogen peroxide (1 μmol), and CPO (1.2 μg) in 1 ml 100 mM phophate buffer. The points represent average of duplicate determinations.


A.
Relative activity (%).
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
pH


B.
Oxidized phenol (%).
100
80
60
40
20
0
1
2
3
4
5
6
7
8
pH

reaction. Unlike TMPD (section 3.3.1.1), the addition of phenol to the reaction mixture had no detectable effect on the buffer pH, therefore the buffer preparation and the activity profile study with phenol were easily carried out. The profile was obtained by estimating the amount of oxidized phenol from the residual phenol, after the reaction was stop by immersion in a 70°C water bath. This profile indicated an optimum activity at pH 3.0, and an appreciable oxidative activity expressed over the entire pH range tested, from 2.75 to 7.0 (Figure 3.18B). At pH 7.0, CPO activity was 65% of the maximum detected at pH 3.0.

3.3.2 Chloroperoxidase stability studies.

Long term storage stability studies with soluble CPO indicated that, in the absence of substrates, the enzyme was stable at 22°C over a range of pH 3.5 to 5.5 (Pickard and Hashimoto 1988). The following studies analyzed the effect of pH on CPO during storage at 5°C.

3.3.2.1 Storage stability.

Because the conditions for maximum catalytic rates are not necessarily optimal for enzyme stability, the investigation of the stability of CPO to pH was initially carried out in the absence of its substrates. Both soluble and immobilized CPO were stored at 5°C, at different pH values. After a 2-week storage period, the two enzyme preparations displayed good stability over a broader pH range, from 3.0 to 6.0 (Figure 3.19), and more than 85% of the initial activity was retained.

The effect of storage time at different pH was also monitored through periodic sampling and assay of the remaining activity (Figure 3.20). No significant difference in stability was observed between the soluble and immobilized enzyme, although the data from the soluble enzyme indicated less variations during the course of the study. The

Figure 3.19. pH stability profiles of CPO during storage at 5°C.

Both soluble (o) and immobilized (♦) CPO were stored in 100 mM phosphate buffer, at different pH values (from 3.1 to 7.0). The activity of the enzyme was determined in a 5-minute CPO oxidation assay at pH 6.0, after a 2-week storage at 5°C. The relative activity was determined from the initial activity of the soluble or immobilized CPO at the corresponding pH of storage.

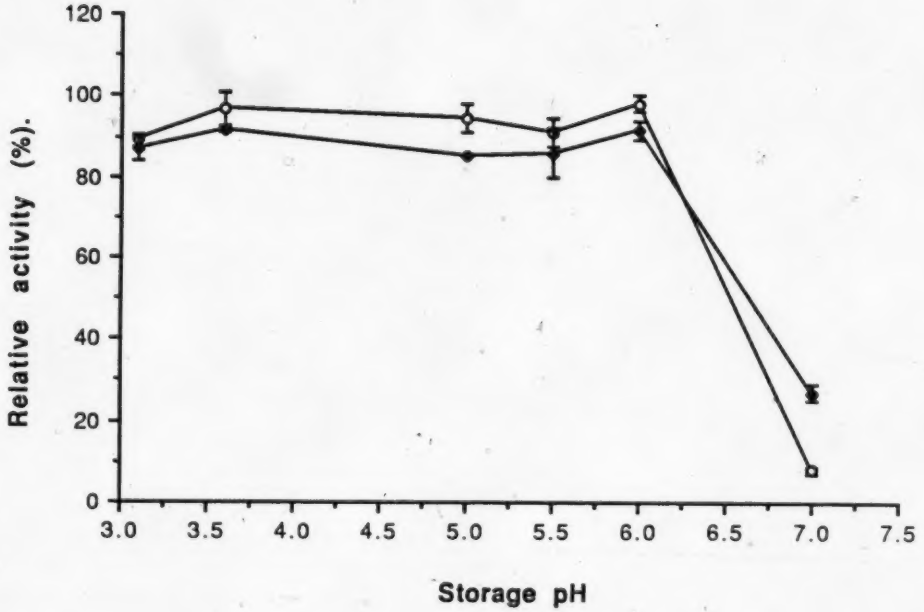
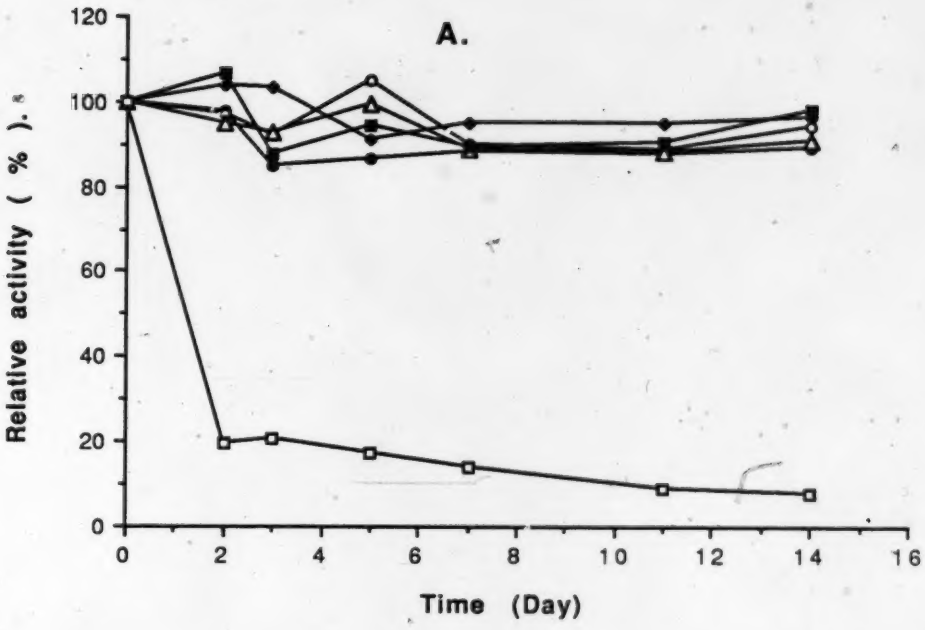
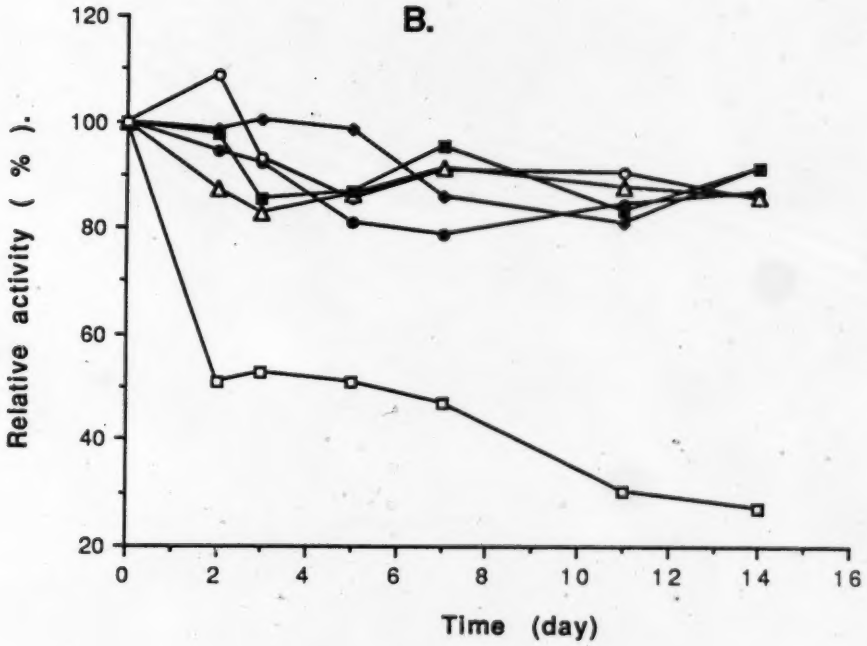

120
100
80
60
40
20
0
Relative activity (%).
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
Storage pH

Figure 3.20. Effect of storage time on the pH-dependent stability of CPO during storage at 5°C.

Both soluble (**A**) and immobilized (**B**) CPO were stored at 5°C in 100 mM phosphate buffer, at different pH values: 3.1 (●), 3.6 (♦), 5.0 (o) 5.5 (Δ), 6.0 (■), and 7.0 (◘). The activity of the enzyme was determined periodically in a 5-minute CPO oxidation assay at pH 6.0.


A.
Relative activity (%).
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
Time (Day)


B.
Relative activity (%).
120
100
80
60
40
20
0
2
4
6
8
10
12
14
16
Time (day)

variation in activity of the immobilized CPO probably arose from some sample loss occurring during the handling, and the difficulty associated with the weighing of the wet immobilized CPO preparation. The results indicated that, within the conditions of the study, both immobilized and soluble CPO were stable over the pH range of 3.0 to 6.0. At pH 7, the enzyme activity was greatly affected within 2 days, and more so for soluble CPO. The immobilization of the enzyme resulted in some stabilization of enzyme activity during the first 5 days of storage at pH 7.0. A 50% retention of initial activity was observed for immobilized CPO, representing about a 2.5-fold increase in stability over the soluble CPO (Figure 3.20). After 4 weeks (results not shown), more than 75% activity was still retained by both soluble and immobilized enzyme in the pH range of 3.1 to 6.0.

Pickard and Hashimoto (1988) reported that, during prolonged periods of storage at 22°C, CPO was more stable within the pH range of 3.5 to 5.5. In this range, CPO retained more than 50% of its activity in 15 days, while only 25% of activity was retained between pH 6.0 and 7.0. The present studies indicated that storage of the enzyme at 5°C extended this pH stability range to 3.1 and 6.0.

3.3.2.2 Operational stability.

The effects of pH and reactants on the catalytic velocity and operational stability of CPO were also investigated during the oxidation of TMPD and phenol. The oxidation of TMPD by CPO was carried out to study the effect of the reactants, H_2O_2 and TMPD, on the retention of activity by the immobilized enzyme, whereas the oxidation of phenol by CPO was carried out to study the effect of H_2O_2 and phenol on the retention of activity by the soluble enzyme during catalysis over a wide range of pH.

3.3.2.2.1 Stability during TMPD oxidation.

The pH-dependent stability of soluble CPO during catalysis was determined from the amount of enzyme activity retained in a series of 5-minute TMPD oxidation assays. Because of the nature of this assay, the product formed could not be easily removed from the reaction mixture. Therefore it was difficult to study the operational stability of CPO with the soluble enzyme. To overcome the product interference, the immobilized enzyme was used. At the end of each reaction, the supernatant was removed and the immobilized enzyme washed thoroughly before fresh reagents were added. The reactions were carried out at room temperature in 100 mM phosphate buffer, at different pH values.

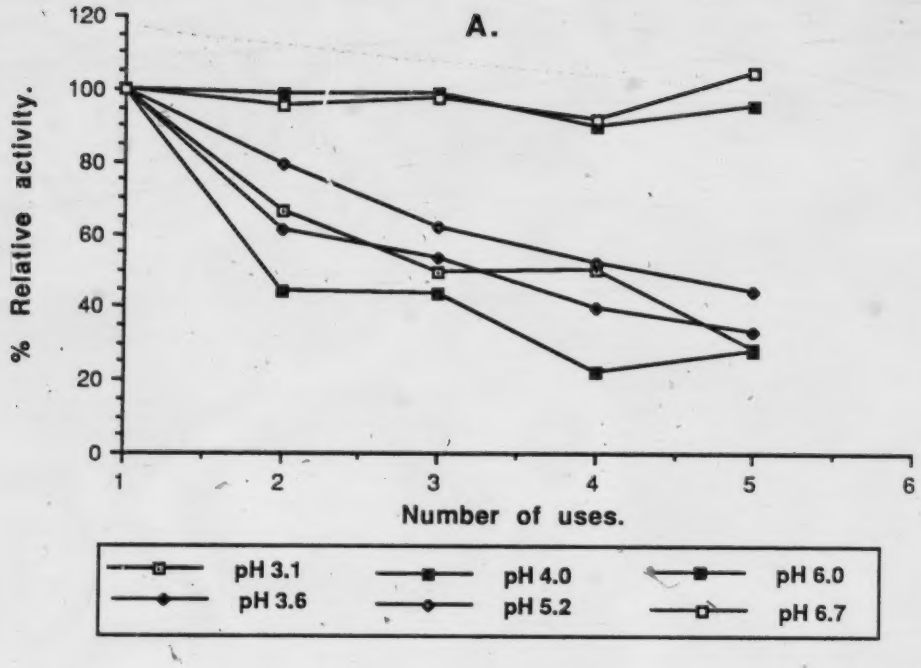
As shown in Figure 3.21A, the immobilized enzyme demonstrated greater stability at pH values near neutralily. After 5 uses, all of the initial activity was retained at pH values between 6.0 and 6.7, and only 30 to 50% of the initial activity retained at pH values below 5.2. Below pH 5.2, the activity was progressively lost with increasing number of uses of the same batch of enzyme (Figure 3.21A).

Figure 3.21B showed the effect of the number of uses on the pH activity profile previously discussed in section 3.3.1.1 and shown in Figure 3.18A. During multiple uses of the immobilized enzyme, a downward shifting of the left side of the profile, from pH 5.2 and lower, was observed. A comparison of the activity profiles from the first and fifth uses indicated a loss in activity at the most acidic pH optimum, being pH 3.1 in Figure 3.21B. No change in the catalytic rate was observed at pH 6.0 and 6.7. Considering the two extreme pH values of the profile, the observed changes reflected the loss of activity at pH 3.1, and the retention of activity at pH 6.7, after 5 uses. From the data in Figure 3.21, it was possible to obtain a stability profile of CPO during operation (Figure 3.22).

The loss of activity in the most acidic pH range may have resulted from the

Figure 3.21. Operational stability of CPO during TMPD oxidation.

The immobilized enzyme was used 5 times to catalyzed the oxidation of TMPD at different pH values. In each use the enzyme was reacted for 5 minutes at room temperature with a mixture containing 1 mM H_2O_2 and 20 mM TMPD in 100 mM phosphate buffer. In between uses, the immobilized enzyme derivative was washed with 100 mM phosphate buffer, pH 5.5. The activity remaining after each use was determined based on the initial activity at the corresponding pH of operation. **A**. The effect of the number of uses on the stability of CPO during the oxidation of TMPD at different pH values. **B**. The effect of the number of uses on the pH-activity profile of CPO during the oxidation of TMPD.


A.
% Relative activity.
120
100
80
60
40
20
0
1
2
3
4
5
6
Number of uses.
pH 3.1
pH 3.6
pH 4.0
pH 5.2
pH 6.0
pH 6.7

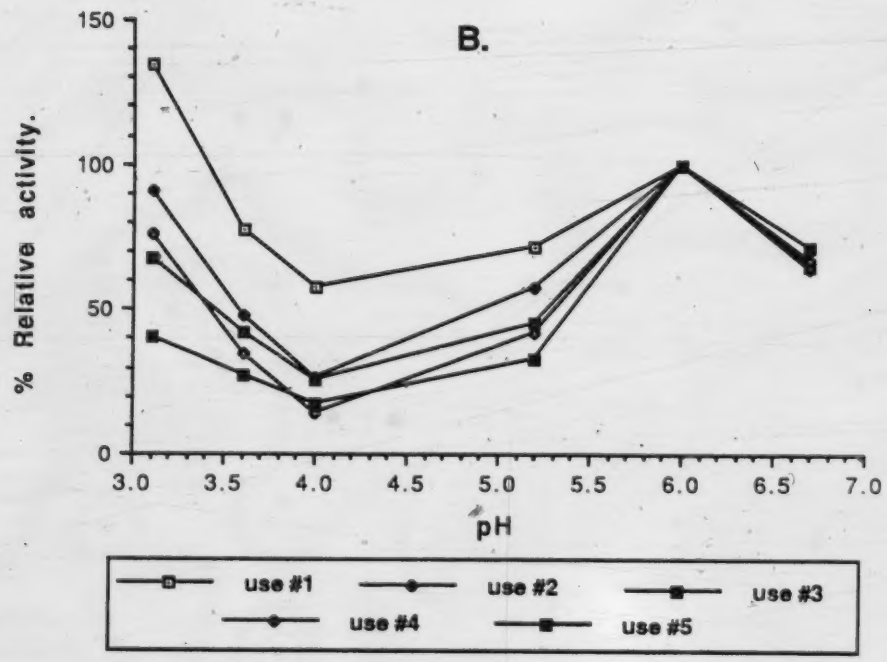
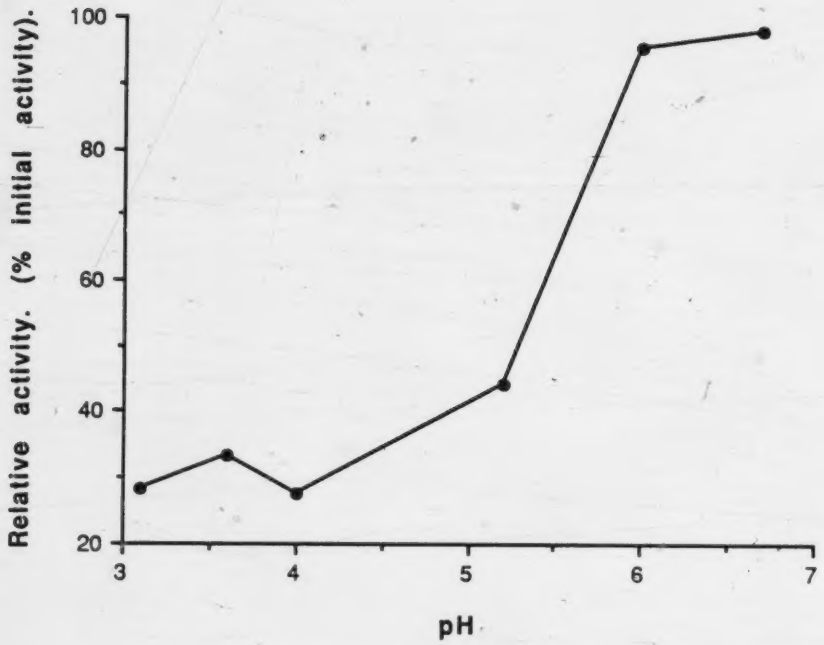

B.
% Relative activity.
150
100
50
0
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
pH
use #1
use #2
use #3
use #4
use #5

Figure 3.22. pH stability profile of CPO during the TMPD oxidation reaction. The immobilized enzyme was used 5 times to catalyze the oxidation of TMPD at different pH values. In each use the enzyme was reacted for 5 minutes at room temperature with a mixture containing 1 mM H_2O_2 and 20 mM TMPD in 100 mM phosphate buffer. In between uses, the immobilized enzyme derivative was washed with 100 mM phosphate buffer, pH 5.5. The enzyme activity was determined at each pH of operation. Based on the activity expressed by the enzyme preparation during the first use, taken as 100% activity in Figure 3.21A, and the remaining activity expressed after the fith use, the profile was obtained.


Relative activity. (% initial activity).
100
80
60
40
20
3
4
5
6
7
pH

inactivation of the enzyme or from the release of the enzyme from the carrier. The instability of the Shiff's base linkage at acidic pH values is well documented (Bernath and Venkatasubramanian 1986; Kennedy and Cabral 1983, 1987). On the other hand, some doubt concerning the crosslinking mechanism between glutaraldehyde and proteins is also well documented. Due to the unusual stability to acids and semicarbazide demonstrated by enzymes immobilized via glutaraldehyde linking (Bernath and Venkatasubramanian 1986), it is believed that the reaction involves several types of products (Goldstein and Manecke 1976, Kennedy and Cabral 1987, Reichlin 1980, Richards and Knowles 1968). Because the activity of soluble CPO decreased considerably at pH 3.0 and 7.0, during storage alone (Pickard and Hashimoto 1988), it can be argued that the loss of activity observed was due to acid and alkaline denaturation of CPO (Lambeir and Dunford 1983b), or inactivation by the reaction products. Further evidence on the decreased stability of a catalytically active CPO at acidic pH values were obtained during the H_2O_2-dependent phenol oxidation studies below.

3.3.2.2.2 Stability during phenol oxidation.

In the previous study, the operational stability of CPO during TMPD oxidation was evaluated with the immobilized enzyme, using a shaking-batch reactor. In this insoluble form, the same batch of enzyme could be recovered at the end of the reaction, washed, and reused a number of times. When this procedure was applied to the oxidation of phenol, the use of immobilized CPO for operational stability studies gave unsatisfactory results (section 3.4).

Therefore, a series of experiments using soluble enzyme were designed, taking advantage of the polymerized nature of the phenol oxidation products (Carmichael *et al.* 1985) and their subsequent removal from the reaction mixture. The CPO-catalyzed

oxidation of phenol was carried out in the presence of hydrogen peroxide, and this reaction resulted in the formation of a yellow color. The reaction was allowed to proceed for a longer period at room temperature. This longer incubation was carried out to ensure the completion of the catalytic reaction and the polymerization process of the oxidized substrate, and to test the stability of the enzyme under the reaction conditions.

The development of a yellow color upon phenol oxidation provided a simple qualitative assay for CPO activity. A transition from yellow to brown color occurred with prolonged incubation of the reaction mixture. This color change was also dependent on the amount of enzyme in the mixture, and the reaction pH. The effect of pH on the color development was observed at low enzyme levels (less than 1 μg CPO). Under these conditions, a constant amount of phenol was oxidized (0.6 μmol) throughout the entire pH range, but brown precipitates formed only at pH 6.5 and 7.0, while soluble yellow products formed at lower pH values. At high enzyme levels (4 μg CPO) brown precipitate formed from pH 3.0 to 7.0. A correlation between the color of the reaction mixture, and the extent of the oxidation and the polymerization reaction was evident.

The operational stability profile of the soluble CPO was studied with the phenol oxidation system. The study of the effect of pH and reactants on CPO activity was carried out by setting four sets of reaction conditions: (i) phenol alone (P), (ii) hydrogen peroxide alone (H), (iii) 1 mM hydrogen peroxide/2 mM phenol (HP 1:2), and (iv) 1 mM hydrogen peroxide/0.5 mM phenol (HP 2:1). In all cases, the enzyme concentration was kept constant (1.2 μg/ml) and the pH range of study was from 2.75 to 7.0.

Initial stability studies with soluble CPO were carried out in the presence of excess hydrogen peroxide (Table 3.9). This series of experiments was termed "HP 2:1", because the initial ratio of hydrogen peroxide to phenol was 2. The reactions were

Table 3.9. Effect of excess of hydrogen peroxide on the oxidation of phenol by CPO, at various pH values.

pH	Total % oxidized phenol	
	Reaction #1[1]	Reaction #2[1]
3.0	80.5	90.9
3.5	83.2	92.5
4.0	83.3	92.7
4.5	84.7	98.1
5.0	79.5	98.4
5.5	78.8	99.0
6.0	74.0	98.5
6.5	74.0	98.4
7.0	68.6	95.4

1) The oxidation of phenol was carried out at different pH values, in the presence of two-fold excess of H_2O_2 as discussed in the results section 3.3.2.2.2, under HP 2:1 series of experiments. The initial reaction mixtures for the reaction #1 contained phenol (0.5 mM), H_2O_2 (1 mM), CPO (1.2 µg/ml), in 70 mM phosphate buffer. The reactions were started with the simultaneous addition of the reactants to the buffers and allowed to proceed to completion at room temperature. The samples were subsequently incubated for 3 days as follows: the first day at room temperatue and the remaining two days at 5°C. This was done to allow the polymerization of oxidation products to go to completion and test the effect of the substrates on the enzymes at various pH values. After the 3-day incubation, the supernatants were recovered and sampled for GC analysis of residual phenol. In an attempt to achieve complete oxidation of phenol (about 96 µM remaining at pH 5.5), more H_2O_2 (0.5 mM) was added to initiate the reaction # 2. After a 1-day incubation at room temperature the total amount of oxidized phenol was again determined from residual phenol as described above.

started with the simultaneous addition of both phenol and hydrogen peroxide to the enzyme and allowed to proceed at room temperature, until one of the substrates was depleted. Although the time required to complete the oxidation reaction was less than 5 minutes, the samples were allowed to stand at room temperature for one day to ensure the completion of the polymerization process and test the stability of the enzyme in the presence of its substrates. After the 24-hour incubation at room temperature, the samples were stored at 5°C for an additional two days. The reaction mixtures were subsequently centrifuged to separate the polymerized oxidation product from the supernatant, and to the supernatant was added the substrate required to start the reaction again, after aliquots of the mixture were analyzed for phenol oxidation. The addition of the required substrate initiated a new round of reaction. In most cases the required or the depleted substrate was found to be hydrogen peroxide. The procedure described above was repeated until the phenol in the reaction mixture was completely oxidized or no enzyme activity was detected with further additions of hydrogen peroxide.

The results in Table 3.9 indicated a slightly higher total amount of phenol oxidized (83.2-84.7%) from pH 3.5 to pH 4.5, followed by a decrease with increasing pH. During this first round of reaction, and despite the use of a two-fold excess amount of hydrogen peroxide, 14 to 32% phenol remained in solution, suggesting that the stoichiometry of the reaction was not 1 to 1, or the consumption of peroxide involved more than one reaction type. In an attempt to achieve a complete oxidation of phenol, more H_2O_2 was added. During this second round of reaction, over 98% of phenol was oxidized in the pH range of 4.5 to 6.5. A shift in the maximum phenol oxidation levels from acidic pH range in the first round of reaction to near neutral pH values in the second round was also observed. What caused this shift?

To answer this question, HP 1:2 series experiments were carried out, in which phenol was used in excess. Under these conditions, 30 to 39% phenol was oxidized

after the first round of reaction (Table 3.10). The highest oxidation level was obtained at pH 3.0. In subsequent rounds of reaction, no response to hydrogen peroxide additions was detected at pH 2.75. As a general pattern, with each new round of reaction, there was a reduction in the range of pH for which the activity was expressed in response to H_2O_2 additions, and this effect was more pronounced at pH values below 4.5. However, a constant amount of phenol (28 to 30%) was oxidized during each of the first three rounds of reaction, in the pH range of 5.0 to 7.0.

To determine the cause of the lack of response to H_2O_2 additions in the acidic pH range, H-1 and P-1 experiments were carried out. In this study, the enzyme was preincubated with 1 mM of each of the two substrate, H_2O_2 or phenol. In these experiments, a complex pattern of activity expression was observed. When the enzyme was preincubated with phenol before starting the reaction with the addition of the hydrogen peroxide, the activity was expressed throughout the entire pH range tested, from pH 3.0 to pH 7.0 (Table 3.11). The total amount of phenol oxidized ranged from 60.4 to 71.6%. The highest oxidation level was obtained at pH 4.5. A comparison of the apparent "activity profiles" determined from the residual phenol in the first rounds of reaction, between the preincubation studies (Table 3.11) and the simultaneous substrate addition studies (Tables 3.9 and 3.10), indicated that the preincubation of the enzyme with phenol resulted in a profile almost similar to that observed during the simultaneous substrate addition studies.

However, the preincubation of the enzyme with H_2O_2 gave three activity profiles, after the addition of phenol, more CPO, and more hydrogen peroxide (Table 3.11, and Figure 3.23). Two of these profiles were almost complementary. In the first case, with the addition of phenol alone [H-1(P) series], no activity was expressed throughout the entire pH range, suggesting that either the enzyme was inactivated or

Table 3.10. Effect of excess of phenol, and incremental additions of hydrogen peroxide on phenol oxidation by CPO at various pH values.

pH	Total % oxidized phenol			
	Reaction #1(1)	Reaction #2	Reaction #3	Reaction #4
2.75	29.7	31.2	30.6	31.0
3.0	38.8	44.3	44.6	43.6
3.5	35.2	58.5	55.8	54.6
4.0	35.3	60.2	65.3	65.0
4.5	35.5	63.5	88.8	90.0
5.0	34.0	63.7	93.1	98.5
5.5	34.0	60.8	92.7	99.4
6.0	28.3	58.3	90.6	99.4
6.5	31.7	59.2	88.7	99.5
7.0	28.5	52.3	83.0	82.3

(1) The oxidation of phenol was carried out at different pH values, as discussed in the results section 3.3.2.2.2, under HP 1:2 series of experiments, in which the phenol:H_2O_2 ratio was 2. The initial reaction mixtures for the reaction #1 contained phenol (1 mM), H_2O_2 (0.5 mM), CPO (1.2 µg/ml), in 70 mM phosphate buffer. The reactions were started with the simultaneous addition of the reactants to the buffers and allowed to proceed to completion at room temperature. The samples were subsequently incubated for 3 days and treated as described for HP 2:1 experiments in the legend to Table 3.9, and phenol oxidation measured for the reaction # 1. In an attempt to achieve complete oxidation of phenol (about 0.66 mM remaining at pH 5.5), more H_2O_2 (0.5 mM) was added to initiate the reaction # 2. After a 1-day incubation at room temperature the total amount of oxidized phenol was again determined from residual phenol. In the reactions (reactions # 3 and 4), more H_2O_2 was added to final concentrations of 0.8 and 1.0 mM, respectively, to oxidize residual phenol in the reaction mixtures.

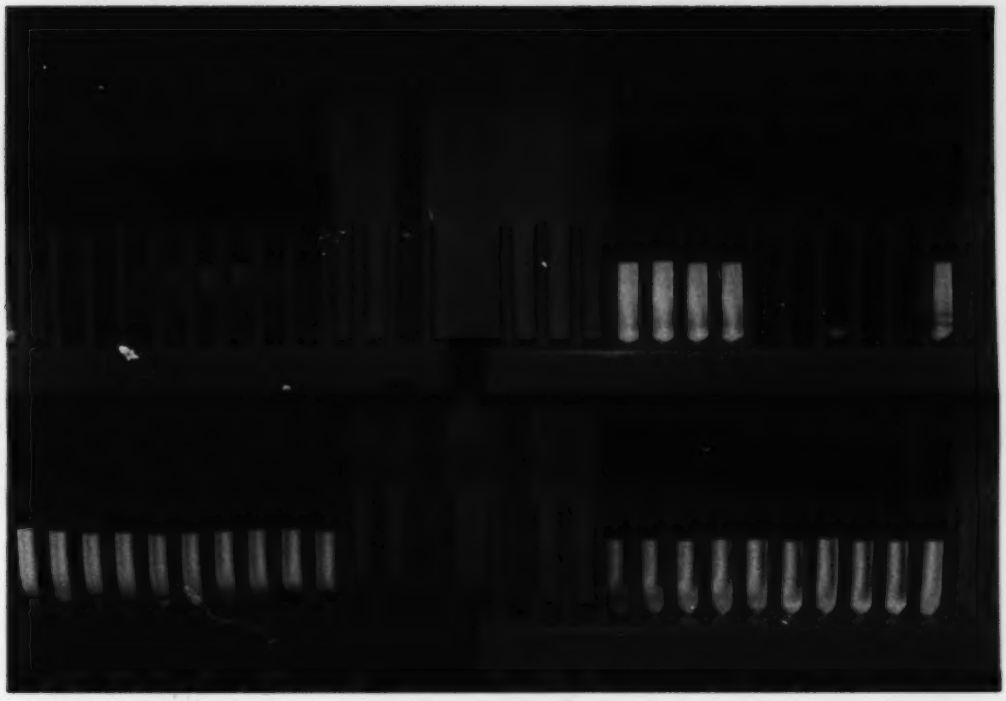
Table 3.11. Effect enzyme preincubation with hydrogen peroxide or phenol on the oxidation of phenol by CPO at various pH values.

pH	Total % oxidized phenol			
	H-1 series[1]			P-1 series[1]
	H-1(P)	H-1(HP)	H-1(CP)	P-1(H)
2.75	0	0	23.1	59.5
3.0	0	0	10.8	62.6
3.5	0	0	0	70.5
4.0	0	0	0	67.0
4.5	0	60.6	0	71.6
5.0	0	63.6	0	70.8
5.5	0	64.5	0	56.1
6.0	0	60.8	0	63.4
6.5	0	58.7	0	62.4
7.0	0	24.5	0	63.5

(1). CPO (1.2 µg/ml) was preincubated for 3 days, as described in legend to Table 3.9, with H_2O_2 (1 mM) (H-1 series) or with phenol (1 mM) (P-1 series), in 70 mM phosphate buffer, at different pH values. After the incubation, H-1 samples were divided in three sets. To one set [H-1(P)], phenol (1 mM) was added, to the second [H-1(HP)], phenol (1 mM) and more H_2O_2 (1 mM) were added, and to the third [H-1(CP)], phenol (1 mM) and more CPO (1.2 µg/ml) were added. To P-1 series, H_2O_2 (1 mM) was added to initiate the reaction. The reactions were allowed to proceed to completion at room temperature. The samples were subsequently incubated for 1 day at room temperatue. This was done to allow the polymerization of oxidation products to go to completion and test the effect of the substrates on the enzyme at various pH values. After the incubation, the supernatants were recovered and sampled for GC analysis of residual phenol, which reflected the activity and stability of the enzyme.

Figure 3.23. Reaction mixtures of the CPO-substrate preincubation experiments (H-1 and P-1 series).

CPO (1.2 μg/ml) was preincubated at different pH values with phenol (1 mM) (P-1 series) or hydrogen peroxide (1 mM) (H-1 series) for 3 days in 70 mM phosphate buffer, as described in the legend to Table 3.9. The pH values tested in the 4 sets of test tubes were, from left to right, 2.75, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0. Top left set: the enzyme was preincubated with phenol and H_2O_2 was added to the mixture. Oxidation activity is detected as a brown product is formed throughout the entire pH range tested. Bottom left set: the enzyme was preincubated with H_2O_2 and phenol was added to the mixture. No activity is detected in the pH range tested. Top right set: the enzyme was preincubated with H_2O_2, and phenol and more H_2O_2 were added. Activity was detected from pH 4.5 to 6.5, with slight activity detected at pH 7.0. And bottom right set: the enzyme was preincubated with H_2O_2, and phenol and more CPO were added. Increasing activity was detected from 3.0 to 2.75.



H_2O_2 was depleted during the preincubation. In the second case, with the addition of phenol and more H_2O_2 [H-1(HP) series], the expression of the activity was now detected from pH 4.5 to 6.5, and about 50% of activity expressed at pH 7.0. Suggesting that, during the storage with H_2O_2, enzyme inactivation did occur at pH values below 4.5; in the pH range of 4.5 to 6.5, enzyme inactivation did not occur, except for partial inativation at pH 7.0, presumably due to alkaline denaturation of the enzyme (Lambeir and Dunford 1983b). However, during the preincubation, H_2O_2 was depleted in the pH range of 4.5 to 7.0, presumably due to the catalatic activity of CPO. Finally, with the addition of phenol and more CPO [H-1(CP) series], the activity was detected this time at the most acidic pH values, and decreased with increase in pH from 2.75 to 3.0. At pH 3.5 and higher, no activity was detected. Suggesting that at pH 2.75 and 3.0, some residual amount of H_2O_2 was present after the preincubation. These results also indicated that between pH 3.0 and 4.5, enzyme activity and H_2O_2 were lacking from the system. The results obtained with the H-1 and P-1 series of studies can be vizualized in Figure 3.23.

The loss of enzyme activity observed in the acidic pH range during the simultaneous addition of both substrates was presumably a product of two phenomena: the acid denaturation of CPO, on one hand (Lambeir and Dunford 1983b), and the effect of H_2O_2 on the enzyme, on the other hand. At pH 3.0 and below, Lambeir and Dunford (1983b) reported that CPO undergoes an irreversible acid denaturation. Since the preincubation studies of enzyme and H_2O_2 indicated that H_2O_2 was not completely depleted from the mixture at pH 2.75 and 3.0, the enzyme must have been rapidly denatured under these conditions. The amount of the remaining H_2O_2 would then be a

reflection of the competition between enzyme inactivation and H_2O_2 depletion via the catalase activity of CPO. The lower the pH, the faster the denaturation rate, and the more H_2O_2 remaining in solution.

It was also observed that, during the experiments where simultaneous addition of both substrates was made, significant activity was expressed at pH below 4.5. The reason for this may be that, in a short time assay, where the initial reaction rates are measured at the optimum pH of activity, the catalytic velocity predominates the inactivation rate. In other words, at pH 2.75-3.0, a high enzyme turnover of CPO takes place before complete inactivation is observed, and valid kinetic data can be obtained.

3.3.2.3 Thermal stability.

The temperature stability profiles of soluble and immobilized CPO were determined by preincubating the enzyme for 1 hour at different temperatures, ranging from 25 to 100°C. The remaining activity at each temperature was determined and compared to the activity of the 25°C samples of the soluble or the immobilized CPO, which remained unaffected by the treatment. Duplicate samples were used and the experiments were repeated at least twice.

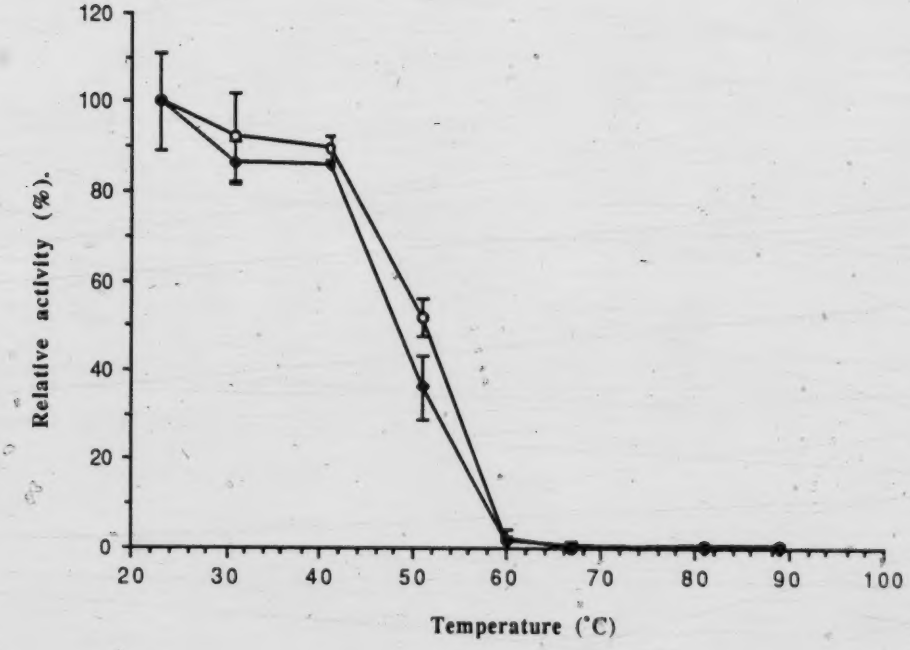
The thermal stability study did not indicate any significant effect on the stability of CPO to temperature upon immobilization. In both forms, CPO lost complete activity after 1 hour of incubation at 60°C (Figure 3.24). However, at temperatures below 40°C, the enzyme retained over 85% of the initial activity in comparison to the room temperature treated sample.

3.3.3 Kinetic constants.

The kinetic constants of CPO were determined during the H_2O_2-dependent

Figure 3.24. Temperature effect on the stability of CPO during storage.

Both soluble (o) and immobilized (♦) CPO were preincubated for 1 hour at different temperatures, in 100 mM phosphate buffer, pH 5.5. At the end of the incubation, the remaining activity of the enzyme was determined in a 5-minute CPO colorimetric assay, at pH 6.0.


120
100
80
60
40
20
0
Relative activity (%).
20
30
40
50
60
70
80
90
100
Temperature (°C)

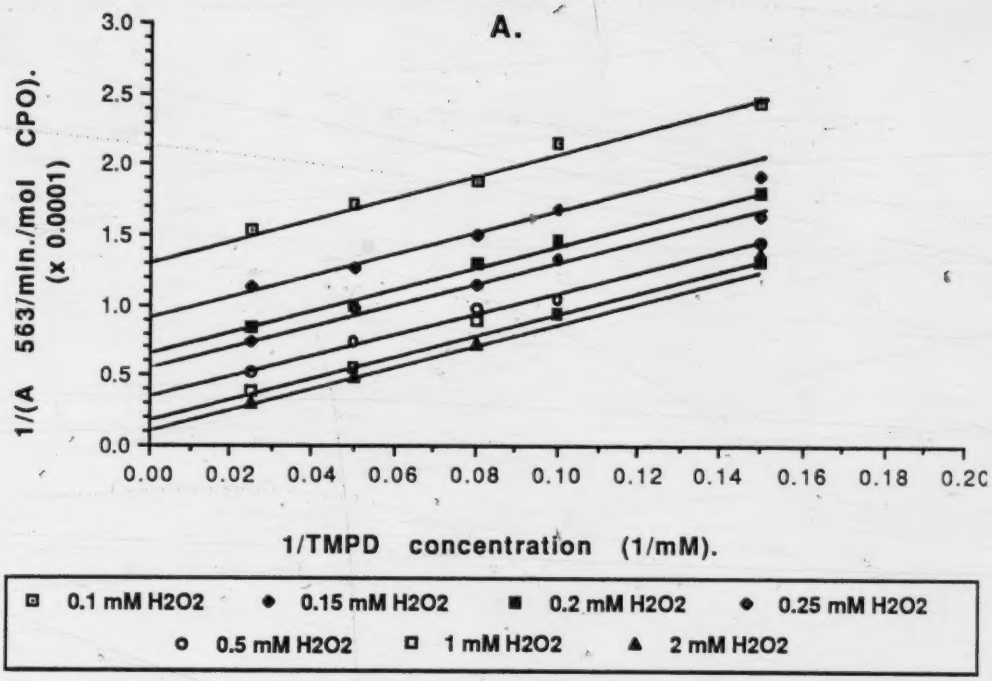
oxidation of TMPD. The reactions were carried out at room temperature in 100 mM phosphate buffer, pH 6.0. To obtain the Km value for H_2O_2, the reciprocals of TMPD concentration were first plotted against the reciprocals of the reaction velocity. By holding the initial concentration of H_2O_2 constant and varying the initial concentration of TMPD, then choosing another initial concentration of H_2O_2 to hold constant while the TMPD concentration was varied, and so on, parallel lines were obtained (Figure 3.25A). The lines shifted downward with increasing TMPD concentrations. The intercepts of the first plot were then replotted against the reciprocals of H_2O_2 concentration in order to derive the Km value for H_2O_2. Generally with enzymes obeying the normal ping-pong reaction mechanism, the limiting Km value for the substrate would be determined from the intercept of the replot curve with the x-axis, and the maximal catalytic velocity determined from the intercept of the replot curve with the y-axis (Segel 1975). However, the replot curve obtained with CPO intercepted these two axes at zero (Figure 3.25B). In this case the Km for H_2O_2 corresponded to infinity.

Similarly, to obtain the Km value for TMPD, the reciprocals of H_2O_2 concentration were first plotted against the reciprocals of the reaction velocity obtained at different TMPD concentrations (Figure 3.26A). Parallel lines were again obtained, and these lines shifted downward with increasing TMPD concentrations. As was the case with H_2O_2, the intercepts of the first plot were replotted against the reciprocals of TMPD concentration, and the Km value for TMPD, derived from the intercept of the curve with the x-axis, was again infinity (Figure 3.26B). The results indicated that the Km of CPO for both sustrates could not be determined.

Although the results from the kinetic study, lead to the formulation of unusual

Figure 3.25. Determination of Km value for hydrogen peroxide.

A. Double reciprocal plots of of the initial rate of hydrogen peroxide-dependent TMPD demethylation by CPO. The initial rates were determined by the colorimetric assay. The 5-minute reactions were carried out at room temperature, in the presence of 0.05 µg CPO in 1 ml 100 mM phosphate buffer, pH 6.0. Initial TMPD concentration was varied systematically (from 5 to 40 mM) at constant initial concentrations of hydrogen peroxide (0.1, 0.15, 0.2, 0.25, 0.5, 1.0, and 2.0 mM). Parallel lines were obtained. **B**. Replot of the intercepts from the double reciprocal plots in (**A**) with the reciprocals of hydrogen peroxide concentration. Points displayed are averages of 4 determinations.


A.
1/(A 563/min./mol CPO).
(x 0.0001)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.00 0.02 0.04 0.06 0.08 0.10 0.12 0.14 0.16 0.18 0.20
1/TMPD concentration (1/mM).
0.1 mM H2O2
0.15 mM H2O2
0.2 mM H2O2
0.25 mM H2O2
0.5 mM H2O2
1 mM H2O2
2 mM H2O2

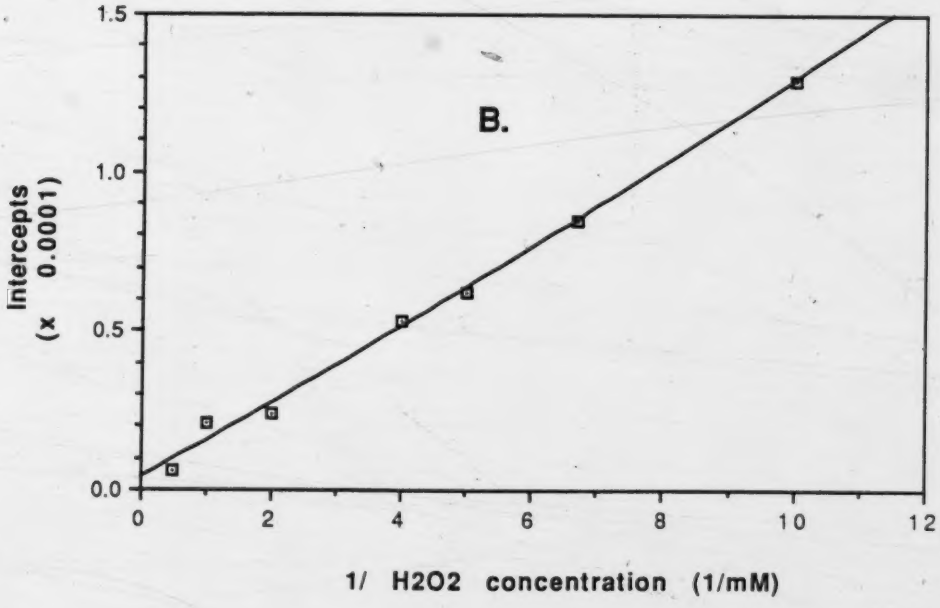
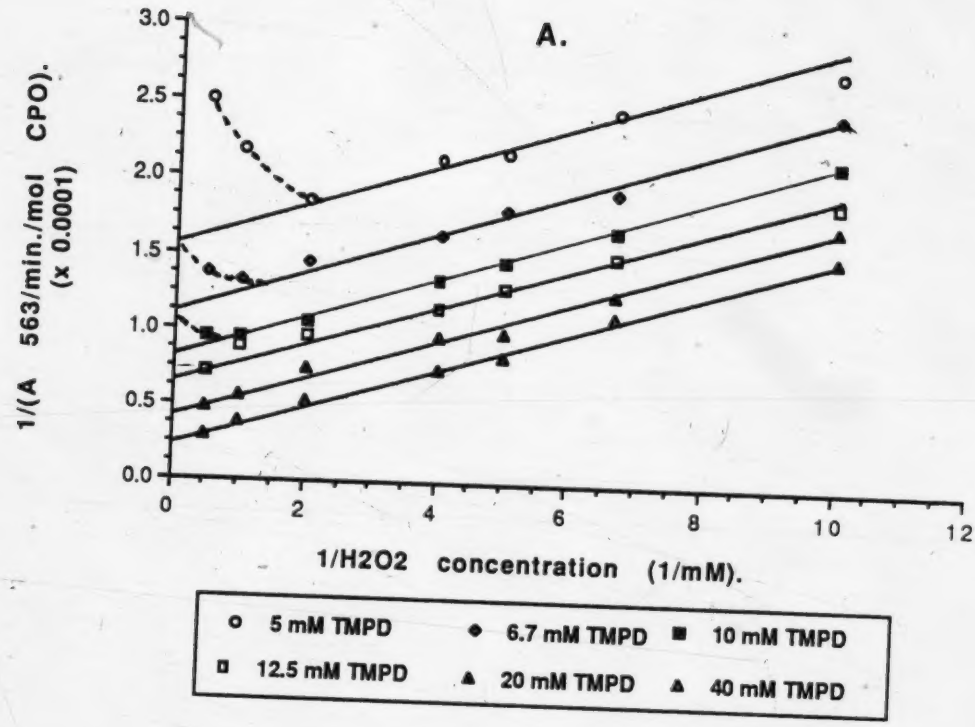
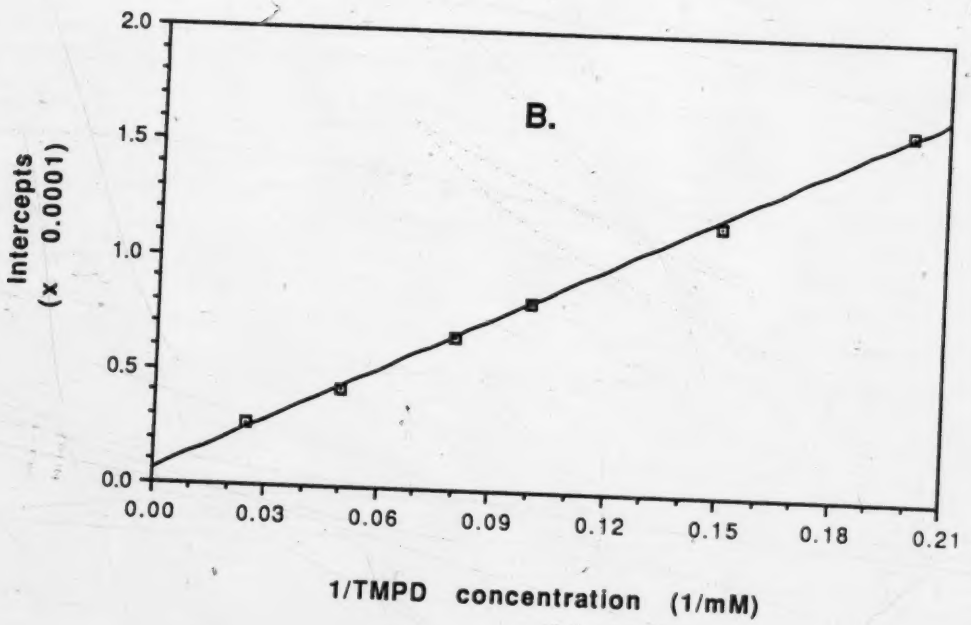

B.
Intercepts
(x 0.0001)
1.5
1.0
0.5
0.0
0 2 4 6 8 10 12
1/ H2O2 concentration (1/mM)

Figure 3.26. Determination of Km value for TMPD.

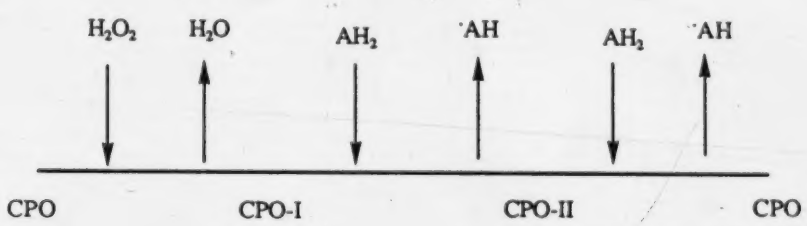
A. Double reciprocal plots of of the initial rate of hydrogen peroxide-dependent TMPD demethylation by CPO. The initial rates were determined by the colorimetric assay. The 5-minute reactions were carried out at room temperature, in the presence of 0.05 μg CPO in 1 ml 100 mM phosphate buffer, pH 6.0. Initial hydrogen peroxide concentration was varied systematically (from 0.1 to 2.0 mM) at constant initial concentrations of TMPD (5, 6.7, 10, 12.5, 20, and 40 mM). Parallel lines were obtained. **B**. Replot of the intercepts from the double reciprocal plots in (**A**) with the reciprocals of TMPD concentration. Points displayed are averages of 4 determinations.


A.
1/(A 563/min./mol CPO).
(x 0.0001)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
1/H2O2 concentration (1/mM).
5 mM TMPD
6.7 mM TMPD
10 mM TMPD
12.5 mM TMPD
20 mM TMPD
40 mM TMPD


B.
Intercepts
(x 0.0001)
2.0
1.5
1.0
0.5
0.0
0.00
0.03
0.06
0.09
0.12
0.15
0.18
0.21
1/TMPD concentration (1/mM)

conclusions, they seemed, however, to agree with the postulated reaction mechanism of CPO and peroxidases in general. This is a "modified ping-pong" reaction mechanism (Dunford, personal communication), in which there is no reversibility of the reaction steps, and the rate constant of the second reaction step (compound II formation) is much larger than that of the third step (regeneration of the native enzyme).

The reaction mechanism can be depicted as follows:

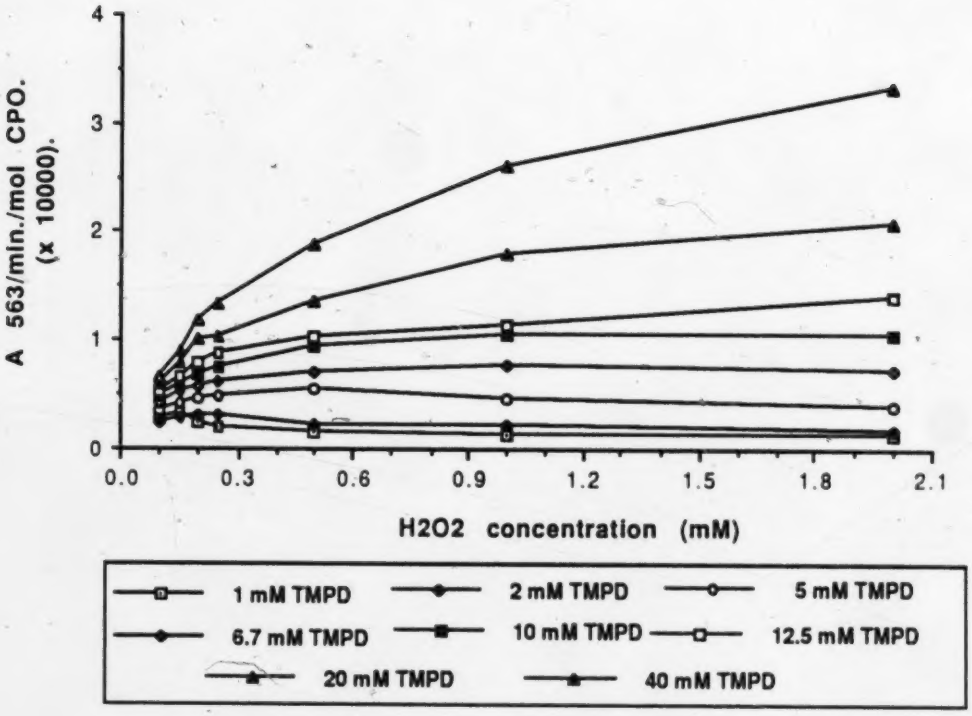


Where AH_2 is the reducing substrate, and ·AH is the free radical; and CPO, CPO-I, and CPO-II correspond to the native enzyme, compound I , and compound II, respectively.

Evident from the kinetic studies was the effect of excess substrate on the catalytic velocity. For clarity, the same kinetic data were plotted differently. This time, the activity or the catalytic velocity was plotted directly against the substrate concentration. An inhibitory effect of H_2O_2 was observed at low TMPD concentrations, and was overcome as the TMPD concentration was increased (Figure 3.27). This effect could be resulting from the conversion of CPO to a less catalytically form in the presence of excess H_2O_2 and absence of a reducing substrate. The implication of this inhibitory effect is greater when kinetic constants are being derived from the double reciprocal plots. As seen in Figure 3.26A, the curves obtained at higher H_2O_2 concentrations deviate from the expected parallel lines, obtained in the

Figure 3.27. Effect of increasing TMPD concentration on the inhibitory effect of CPO activity at high concentration of H_2O_2.

The relationship was determined by carrying out the hydrogen peroxide-dependent TMPD demethylation by CPO. The initial rates were determined in 5-minute reactions, at room temperature, in the presence of 0.05 µg CPO in 1 ml 100 mM phosphate buffer, pH 6.0. Initial hydrogen peroxide concentration was varied systematically (from 0.1 to 2.0 mM) at constant initial concentrations of TMPD (1, 2, 5, 6.7, 10, 12.5, 20, and 40 mM).


A 563/min./mol CPO.
(x 10000).
4
3
2
1
0
0.0
0.3
0.6
0.9
1.2
1.5
1.8
2.1
H2O2 concentration (mM)
1 mM TMPD
2 mM TMPD
5 mM TMPD
6.7 mM TMPD
10 mM TMPD
12.5 mM TMPD
20 mM TMPD
40 mM TMPD

absence of H_2O_2 inhibition at low concentrations of this substrate.

3.4 OXIDATION OF PHENOL BY IMMOBILIZED CPO.

Extensive studies on the oxidative activity of HRPO have revealed a number of potential uses for peroxidases. HRPO has been shown to efficiently carry out the removal of carcinogenic aromatic amines from industrial aqueous effluents (Klibanov and Morris 1981), and phenols from coal-conversion wastewaters (Klibanov et al. 1983). CPO has also been reported to oxidize phenolic compounds in the absence of halide ion (Carmichael 1987, Carmichael et al. 1985). In the present study, the potential of immobilized CPO in the removal of phenol from aqueous solutions was tested.

The initial studies on phenol oxidation by immobilized CPO were carried out in a shaking-batch reactor. These studies were only preliminary, and were not repeated many times. The use of the shaking-batch immobilized CPO reactor gave unsatisfactory results. The immobilized enzyme derivative was losing its activity during the first use, as a result of the irreversible interaction of the immobilized enzyme material with the oxidation products. It was observed that during the course of the reaction, the 40-mesh-glass immobilization carrier collected on its surface a yellow-brown colored product produced during the oxidation of phenol. To overcome this inhibitory effect, the contact time between the immobilized enzyme and the oxidation products needed to be reduced. This was partially achieved by using a packed-column immobilized CPO reactor, for preliminary results (not shown) indicated an increase in the enzyme operational lifetime.

4. DISCUSSION.

4.1 Immobilization of chloroperoxidase on glass beads.

Fast, efficient utilization of reactants and an extended operational lifetime are among the ultimate requirements for the commercial use of an enzyme (Neidleman and Geigert 1986). Several studies have been reported dealing with the mechanisms of enhancing the catalytic velocity of CPO and other peroxidases. These include the careful balancing of the pH, the levels of hydrogen peroxide, and halide ion in the reaction mixture; the supply of additives; and the use of alternative reaction solvents. But maximal catalytic velocity is only part of the concern in commercial applications. It must be associated with efficient utilization of reactants, enhancement of the operational lifetime of the biocatalyst, and efficient downstream processing.

At the beginning of this project there was only one reported study on the immobilization of CPO: the patent on the method for producing iodine, using CPO adsorbed on DEAE-Sephadex A-50 beads (Neidleman and Geigert 1981). However, the immobilization of a number of peroxidases had been reported (Neidleman and Geigert 1986, Nobuya *et al.* 1987). Of a particular interest to my work was the study by Olsson and Ögren (1983) on the covalent attachment of HRPO on derivatized glass beads, because the immobilization method and carrier they used were suitable for this project. Therefore, the procedure described by Olsson and Ögren (1983), which was initially described by Weetall (1976), was applied to CPO immobilization on glass beads. As previously stated, the three major steps of this procedure were: the amination of glass, the glutaraldehyde treatment of aminated glass, and the attachment of enzyme to the derivatized glass.

The amination of the glass beads was carried out according to the established methodology, with no significant changes. Attempts were not made to estimate the

amount of amino groups attached to the glass beads, although assays for direct quantitation of amino groups covalently bound to solid supports have been described (Brotherton *et al.* 1976, Buchholz and Klein 1987). Nevertheless, it was possible to evaluate the optimum amination reaction conditions on the basis of the activity of bound enzyme. The amination experiments indicated that the silanization of glass beads in aqueous solvent resulted in higher loading of amino groups than in organic solvent. The mechanism by which the solvent affects the extent of the amination of glass is not understood. Olsson and Ögren (1983) found that the organic amination procedure introduced a larger amount of amino functional groups than the aqueous amination procedure, whereas Weetall (1976) found that the latter resulted in a slightly more stable product.

The treatment of aminopropyl-glass with glutaraldehyde was also carried out according to the established methodology (Weetall 1976). The results from the time course study indicated that the binding of CPO to aminated glass did not require the glutaraldehyde pretreatment step. However, when this step was carried out, a shorter treatment period resulted in higher bound activity; a prolonged treatment resulted instead in a decrease of bound activity. These observations lead to a number of conclusions.

1) CPO could bind directly to aminopropyl-glass. This binding appeared to involve adsorptive forces and ionic interactions, with the later being a predominant binding mechanism. Evidence supporting this conclusion came from the amino acid (Morris and Hager 1966) and peptide sequence (Fang *et al.* 1986, Kenigsberg 1987) analyses, and the known isoelectric points (Hashimoto and Pickard 1984, Pickard and Hashimoto 1982, Sae and Cunningham 1979) of CPO. CPO has a predominence of acidic residues over the basic ones, and they represent about 10% of the total amino acid content. The isoelectric points of the isoenzymes produced by *C. fumago* are in

the range of 3.6 to 3.9. As a result, CPO is an acidic enzyme, with a net negative charge at pH values above 3.9, and under the immobilization conditions used in these studies. Further evidence in support of the ionic nature of the binding of CPO to aminopropyl-glass was provided by the studies on the effects of the buffer concentration and pH on the immobilization of CPO on aminated controlled-pore glass. The results indicated the dependence of the binding on the pH and ionic strength. The enzyme binding to aminpropyl-glass decreased with pH from neutrality, with virtually no binding at pH values below 4.0, and decreased with increasing ionic strength.

2) The pretreatment of aminopropyl-glass with glutaraldehyde decreased the amount of binding sites available for the attachment of the enzyme on the carrier. The established mechanism of the reaction between enzymes and glutaraldehyde involves the formation of an aldimine bond (Kennedy and Cabral 1987). Basic residues such as lysines are required for the linkage. Theoretically, because of its low content of basic residues - only 6 L-lysines (Kenigsberg *et al.* 1987) - CPO should not be amenable to immobilization coupling reactions that involve amino groups. However, the present study indicated that enzyme loading occurred by some of these reactions. The interpretation of the time course experiment data with the glutaraldehyde linker method could be made in the light of stabilization and competition effects. During the shorter treatment of aminopropyl-glass with glutaraldehyde, a small fraction of the amino functional groups presumably reacted with glutaraldehyde. The subsequent treatment of this material with an enzyme solution resulted in the ionic attachment of CPO to the remaining amines on the glass. The interaction between the few lysines of the ionically-bound enzyme and adjacent glutaraldehyde molecules resulted then in the stabilization of the enzyme during the subsequent washing steps. On the other hand, longer glutaraldehyde treatment appeared to favor glutaraldehyde over the enzyme in the competition for amines on the glass, resulting in a decrease of bound activity.

Obviously an optimum glutaraldehyde treatment would depend on the balance between stabilization and competition effects. The story is complicated by the postulated second mechanism of linkage between enzymes and glutaraldehyde, which involves more functional groups and accounts for the greater stability of the Schiff's bond formed (Goldstein and Manecke 1976, Kennedy and Cabral 1987, Reichlin 1980, Richards and Knowles 1968). In this scheme, the reaction involves several types of products. Some of these are formed from the conjugate addition of enzyme amino groups to ethylenic double bonds of α,β-unsaturated oligomers, contained in the commercial aqueous glutaraldehyde solution. Sulfyldryl and imidazole groups may undergo similar reactions.

3) Since the nature of the linkage formed between enzymes and glutaraldehyde is not fully understood, a conclusion cannot be easily made at this point regarding the suitability of the glutaraldehyde linker method (Olsson and Ögren 1983, Weetall 1976) for CPO immobilization. However, studies carried out with CPO (this thesis) and HRPO (Olsson and Ögren 1983) suggested that the glutaraldehyde linker method is not suitable for these two peroxidases. Olsson and Ögren (1983) reported that HRPO coupled to porous glass by the glutaraldehyde linker method had lower activity and stability than the azo-coupled enzyme. They achieved loadings of 3 mg and as high as 70 mg of protein per g of glass with the glutaraldehyde coupling and azo coupling methods, respectively. In this present study, the immobilization of CPO to arylamine glass by azo coupling was attempted. However, due to some procedural difficulties accountered during the survey experiment, the method was discontinued. Instead, a modified method of the glutaraldehyde coupling technique - the glutaraldehyde crosslinking method - was later developed and applied. With this modification, the enzyme was first concentrated on the aminopropyl-carrier electrostatically, and then later crosslinked and stabilized with glutaraldehyde. The results indicated that higher

coupling yield and activity could be obtained by this method than by the established glutaraldehyde linker method. The immobilized enzyme loading obtained by this modified method was 106 mg CPO/g glass, about 50% higher than that obtained by the azo coupling of HRPO (Olsson and Ögren 1983). At this high loading capacity, the specific activity of immobilized CPO was very low, about 8% the activity of the soluble enzyme. This specific activity could, however, be improved by reducing the amount of enzyme presented to the carrier during the immobilization reaction. Immobilized enzyme preparations with up to 36% of bound enzyme being active were obtained.

Since no specific activity data were reported for HRPO study, a comparative evaluation of this parameter can only be made for unrelated systems. Toldrá *et al.* (1986) carried the immobilization of α-glucoamylase on porous glass, and reported not only data of the effects of the enzyme:carrier ratio on enzyme loading but also the specific activity values of the immobilized enzyme preparations. Their study indicated that the specific activities could be increased from 13 to 20%, by decreasing the enzyme:carrier ratio from 150 to 25 mg protein/g glass. They also found some correlation between the concentration of the amination solution, the enzyme loading, and the specific activity of the immobilized enzyme. Using between 2 and 10% aminopropyltriethoxysilane, they observed a proportional increase in amount of enzyme immobilized and a decrease in the specific activity of the immobilized enzyme. As observed in the present CPO immobilization study, the decrease in activity was presumably due to an increase in multiple bonding between the glass and the protein, which resulted in physical hindrance of the enzyme. High concentration of the reactive groups on the glass and the crosslinking agent can partially or totally occlude the active site of the enzyme due to too many cross-linkages or due to having enzymes molecules too close to each other (Pye and Chance 1976). Leonowicz *et al.* (1988) immobilized the fungal laccase on porous glass beads with 90% coupling yield. This high coupling

yield was, however, achieved at a very low enzyme:carrier ratio of 0.3 mg protein per g of glass. These authors reported that the enzyme in the immobilized form had also retained 90% of its specific activity.

4.2 Immobilized chloroperoxidase assay.

It is evident from the above discussion and from a survey of a number of publications on enzyme immobilization that the activity of an enzyme in the immobilized form is often estimated by the "difference method". A naive assumption is made that the amount of enzyme activity not recovered in the washings is the total activity associated with the carrier. Some of the reasons for adopting this approach to measure the immobilized enzyme activity are: (i) the existing assays are primarily developed for soluble enzyme systems, (ii) a direct determination of bound activity may not be possible with the established enzyme assays unless some modifications are made to these assays, or may require the development of new assays, and (iii) it can be tedious to carry out direct quantitation of bound activity on a routine basis, for special care must be taken during the manipulation of wet cakes of immobilized enzyme. Therefore it is convenient to use existing enzyme assays as they are. Additional problems arise with immobilized enzyme of very high activity. In this case minute multiple samples must be weighed out and activity determined. Unlike the soluble enzyme that can be easily diluted out, with immobilized enzyme, the minimum achievable activity is limited by the sample size that can be accurately weighed out and is capable of being handled in the assay.

Therefore, a preliminary step in this study on CPO immobilization was to find a suitable means for the direct determination of activity of the enzyme bound on the carrier. The measurement of bound activity by the "difference method" would not provide meaningful data for comparative studies of different immobilized enzymes

parameters such as, specific activity, coupling efficiency, different immobilization techniques. This information may also be of economic importance, if an enzyme is to be commercially competitive.

A number of enzyme assays were available at the beginning of this project. Some of them were *a priori* known to be unsuitable, such as the chlorination assay (Morris and Hager 1966); some others could have been modified to assay immobilized CPO activity, such as the colorimetric iodination assay (Hager *et al.* 1966). Also available were some classical peroxidase substrates commonly used as chromogen in peroxidase assays 2,2'-dihydroxy-3,3'-dimethoxy-biphenyl and 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid), known as guaiacol (Saunders *et al.* 1964) and ABTS (Bardsley 1985), respectively. Emphasis was, however, placed on the development of a new assay for two reasons: (i) to evaluate a new CPO:substrate system and (ii) to make some contribution to the present knowledge of CPO.

Tetramethyl-*p*-phenylenediamine (TMPD) was selected as the reducing substrate, although it was a difficult substrate to use for an enzyme assay. TMPD generates a high absorbance background due to its autooxidation in oxygenated aqueous solutions, and it has a relatively low solubility in aqueous solvent. Very little was known on how to stop the oxidation reaction after a specified time, without affecting the developed color. However, this substrate has been used as an artificial electron donor in a manometric assay to detect cytochrome oxidase activity (Jurtshuk *et al.* 1967). In the present study, advantage was taken of the intense blue color developed by TMPD upon its oxidation, and at the same time, steps were taken to minimize its autooxidation rate.

By analogy to the documented oxidation of N,N-dimethylaniline (Kedderis *et al.* 1980), antipyrine (Ashley and Griffin 1981) by CPO, and aminopyrine by other hemoproteins (Griffin and Ting 1978), it is assumed that the mechanism by which CPO

oxidizes TMPD involves the N-demethylation of TMPD in the presence of hydrogen peroxide, resulting in the production of a free radical and formaldehyde. The aromatic amine free radicals can undergo various nonenzymatic reactions, such as dimerization, depending on its chemical structure and the experimental conditions (Ashley *et al.* 1980). The free radicals generated by these N-methyl compounds are unusually stable, and when the coupling reaction is inhibited by a *para* substition, as occurs with TMPD, there is considerable enhancement of the rate of N-demethylation and stability of the free radical (Griffin 1978). The quantitation of the intensity of the color produced by the aromatic amine free radicals was the basis of the CPO peroxidation assay.

The two-pH optima phenomenon observed during the characterization of the assay studies was initially observed by Thomas *et al.* (1970) for the chloride-stimulated evolution of oxygen from hydrogen peroxide, and by Kedderis *et al.* (1980) for the chloride-stimulated demethylation of N,N-dimethylaniline. In both studies, the addition of chloride anions to CPO at low pH (2.5 to 4) stimulated this second activity. It is therefore believed that CPO has two catalytically-active forms, an acidic and a neutral form, and the pKa for the transition from the acidic halide-binding form to the neutral form, which does not bind halide anions is between pH 3 and 5 (Thomas *et al.* 1970). The stimulation by the halide anions, is thought to be due to specific binding of halide anion to the vicinity of the heme active site (Kedderis *et al.* 1980).

A good assay for the quantitation of activity of the enzyme in the immobilized form must be convenient and reproducible; must not affect the stability of the enzyme during the course of the assay; and must have a broad linear response to enzyme concentration. The CPO peroxidation (or demethylation) assay developed during this project had these characteristics. But, it is also appropriate at this stage to acknowledge that measuring the activity of the powder-like porous glass beads of high bound activity by the shaking-batch reactor technique was at times a frustrating and tedious process.

Yet the use of this technique was necessary, if valid data were to be generated that can be compared to soluble enzyme systems, because it allowed the maintenance of homogeneous conditions during the assay. The assay conditions were defined not only to optimize the catalytic velocity but also the stability of the enzyme. In this respect, the classical chlorination assay (Morris and Hager 1966) failed to meet this criterion. The reaction pH of 3.0, selected on the basis of the catalytic activity, has been shown in this thesis to inactivate the enzyme. This inactivation may not be very critical for measuring the rapid initial rate of a reaction catalyzed by soluble enzyme, but its avoidance is essential for an enzyme preparation that should be recovered and reused.

4.3 Kinetics of chloroperoxidase-catalyzed N-demethylation of tetramethyl-*p*-phenylenediamine.

In the original outline of this thesis project, the kinetic studies of CPO were to be included as part of the section on the characterization of the immobilized enzyme. The initial intent was to determine the Michaelis-Menten constants for TMPD and H_2O_2 using the soluble enzyme in order to define the conditions of the enzyme assay, then compare these constants to those obtained with the immobilized enzyme. However, during the course of these studies, a sizeable amount of data was derived from the kinetic studies, necessitating the devotion of a separate discussion section to this topic. The characterization studies on CPO stability under various storage conditions presented in the results section are straightforward and will not be discussed here. The results on operational stability will be discussed later in a section below, along with the phenol oxidation studies.

Peroxidases are difficult enzymes to study by steady-state kinetic methods because of the complexities that can occur during their reactions (Bardsley 1985). The free radical products formed during the reaction can react with the enzyme species

leading to loss of enzyme activity. The possible interaction that can occur among the various enzyme species, can be a source of artifacts in kinetic studies. The kinetics of the peroxidase reaction are often obscured since both reductive steps of compound I to regenerate the native enzyme tend to occur spontaneously even in the absence of the hydrogen donor (Saunders *et al.* 1964), and both compounds I and II are reduced by the same substrate. Therefore caution must be exercised in the interpretation of the steady-state kinetics of peroxidases (Bardsley 1985).

The kinetic studies of soluble and immobilized CPO were carried out as described for peroxidases by Segel (1975), in keeping with the postulated ping-pong kinetic mechanism. The concentrations of both substrates, TMPD and H_2O_2, were varied systematically and the results analyzed assuming steady state conditions, as described in the results section. The replot of the primary double reciprocal plots yielded a straight line intercepting both the vertical and horizontal axes at zero. No Km or Vmax values could be derived from these plots. These experiments, carried out in duplicate, were repeated four times over a several-week period. The data were consistent and indicated that the Michaelis-Menten kinetic constants could not be derived. During these studies, excessive care was taken to control the reaction pH, for varying the TMPD-$(HCl)_2$ concentration was inevitably associated with a change in the pH. At first it appeared that there were some experimental errors associated with this study. But the validity of the data was not confirmed until after a discussion with Dr. H.B. Dunford (Chemistry department, U of A). It was demonstrated that, on the basis of peroxidase kinetic equations (refer to the appendix), no Michaelis-Menten kinetic constants could be derived for CPO, as might be the case with other peroxidases.

This study on CPO kinetics represents the first report of experimental data in support of the postulated "modified ping-pong kinetic mechanism" of peroxidases

(Dunford 1989, Dunford and Stillman 1976). However there have been a number of reports on peroxidases kinetics in which Michaelis-Menten constants have been determined. In some instances, these constants were derived from the primary plots, where only one parallel line was obtained by varying one of the substrate concentration at constant concentration of the other (Hollenberg *et al.* 1974, Kedderis *et al.* 1980). The problem with these studies is that by selecting different concentrations of the second substrate and varying the first substrate concentration, a series of parallel line would be obtained. Thus each line would have different Km and Vmax values. In other cases, valid kinetic studies have been also carried out, where the different kinetic parameters (limiting Km and Vmax) had been derived from the slopes and intercepts of the secondary plots (de Boer and Wever 1988, Kedderis and Hollenberg 1983). In their kinetic studies on CPO-catalyzed N-demethylations, Kedderis and Hollenberg (1983) reported valid kinetic constants for the demethylation reaction obtained from the intercepts of secondary replots. These authors found that the values of kinetic constants derived by this method (Kedderis and Hollenberg 1983) were in good agreement with the apparent kinetic constants for the demethylation reaction previously determined using a single fixed concentration of the second substrate (Kedderis *et al.* 1980). Clearly there is no agreement between these kinetic studies and the present study on CPO, using the methodology developed for the quantitation of immobilized CPO.

4.4 Operational stability of chloroperoxidase.

In the course of its catalytic activity, CPO faces oxidative stress. Hydrogen peroxide, as the substrate, and hypochlorous acid, as the product in chloride-dependent reactions, are two powerful oxidants that may greatly affect the enzyme operational half life (Neidleman 1989). The immobilization of CPO, and the minimization of the effects of these oxidants on the enzyme were proposed to increase the enzyme lifetime

(Neidleman and Geigert 1986). The experiments on H_2O_2-dependent phenol oxidation by soluble CPO were designed to address this second issue without making use of an immobilized enzyme system, and to define the optimum operational conditions for efficient multiple uses of the immobilized enzyme.

The study on the reusability of immobilized enzyme during TMPD oxidation indicated that CPO retained significant activity at pH 6.0 and 6.7. However, at pH 5.2 and lower, the activity decreased with the number of uses. Complementary to this study were the operational stability studies carried with the soluble CPO-phenol oxidation system. Again, the data indicated that, during multiple uses of the same batch of enzyme and in the presence of excess phenol, significant activity was retained at pH values near neutrality, although the enzyme was catalytically more active at low pH values, as observed from a single use. Attempts to carry out similar operational stability studies with immobilized enzyme failed, since the products generated during the oxidation of phenol complexed with the support materials. The results from both TMPD and phenol oxidation studies suggested that (i) the decrease in activity of immobilized CPO, associated with a decrease in pH, was not due to desorption of the enzyme from the carrier, but in part to enzyme denaturation and to the presence of an inhibitory or toxic substance in the reaction mixture; (ii) as a biocatalyst, the immobilized enzyme was not suitable for removal of phenolics from aqueous solutions, in a shaking-batch reactor system.

The loss of enzyme activity observed in the acidic pH range during the simultaneous addition of both substrates was a product of two phenomena: the acid denaturation of CPO, on one hand (Lambeir and Dunford 1983b), and the inactivating effect of H_2O_2 or H_2O_2-degradation products on the enzyme, on the other hand. Lambeir and Dunford (1983b) reported that at pH 3.0 and below, CPO undergoes an

irreversible acid denaturation. Because the preincubation studies of enzyme and H_2O_2 indicated that H_2O_2 was not completely depleted from the mixture at pH 2.75 and 3.0, while activity was lost, the enzyme must have been rapidly denatured under these conditions.

At low H_2O_2 concentration (1 mM), the inactivating effect of H_2O_2 or H_2O_2-degradation products on the enzyme was pH-dependent. Between pH 3.0 to <4.5, the interaction of H_2O_2-degradation products with CPO played a major role in enzyme inactivation. It has been hypothesized that CPO has two catalytically active forms, an acidic and a neutral form (Thomas *et al.* 1970). Under acidic conditions, and in the absence of a reducing substrate, two types of interactions might be taking place between H_2O_2 (and its products) and the acidic form of the enzyme, leading to the formation of abortive and productive complexes. The abortive complex might result from the interaction of H_2O_2-degradation products with an important acidic functional group on CPO. In the protonated form, this functional group might react with H_2O_2-degradation products, leading to an irreversible inactivation of the enzyme, by causing unfavorable conformational changes or interference at the active site. The preincubation studies do not provide evidence for a direct inactivation of CPO by H_2O_2. However, if H_2O_2 was directly involved in the enzyme inactivation, rather than its degradation products, one would expect to see a progressive increase in the retention of activity, as the pH increased from 3.0 to <4.5, because the rate of H_2O_2 removal from the reaction mixture increased, as the rate the catalase activity of CPO increased with pH. This activity was optimal around pH 4.0, as reported by Thomas *et al.* (1970), slightly lower than the pH 4.5, determined during these studies (this thesis).

Instead, a complete depletion of H_2O_2 and inactivation of CPO was observed in this pH range. Implying that, the depletion of H_2O_2 via the catalatic activity proceeded to completion first, and in the second stage of events, the H_2O_2-degradation products then interacted with the acidic form of the enzyme to inactivate it.

What is the nature of these H_2O_2-degradation products? The breakdown of H_2O_2-degradation products gives rise to H_2O and O_2. Obviously H_2O was not toxic to the enzyme. Kohler and co-workers (Huwiler *et al.* 1986, Jenzer *et al.* 1986, Wildberger *et al.* 1986) have studied the stability of lactoperoxidase in the course of I^- oxidation. They demonstrated that the irreversible inactivation of this enzyme correlated with O_2 production, as a result of the pseudocatalytic H_2O_2 degradation in the presence of I_2. They proposed that the singlet oxygen species as the inactivating agent. They also demonstrated that in the presence of H_2O_2 alone and below a critical concentration (0.1 mM) of this substrate, the enzyme was stable.

In the pH range of 4.5 to 6.5, it appears that the stability of the CPO to H_2O_2 was primarily dependent on the concentration of H_2O_2 and the H_2O_2:phenol ratios. In their studies on lactoperoxidase stability, Jenzer *et al.* (1986) assigned the inactivating action of H_2O_2 to hydroxyl radicals formed by a Fenton reaction between the intermediate ferrous state of the enzyme and H_2O_2, at higher concentration of H_2O_2. The attack of these hydroxyl radicals on the heme moiety lead to a ring opening and loss of enzyme activity. One might also envisage a possible role of the catalatic activity of CPO in removing H_2O_2 from the reaction mixture, thereby protecting the enzyme. This protection could, however, be overcome by too much H_2O_2, which would result

in the formation of the less catalytically active form of the enzyme - compound III (Nakajima *et al.* 1985). Presumably, this compound III serves as an intermediate in the inactivation of CPO at high hydrogen peroxide concentration. Early studies on TMPD oxidation (this thesis) indicated that in the presence of 2 mM H_2O_2, the enzyme activity was reduced. Optimum activity was expressed at H_2O_2 concentration of 0.8-1.0 mM. Clearly high H_2O_2 concentration affect the enzyme activity even near neutral pH values. For this reason the maximum H_2O_2 concentration used in these studies did not exceed 1 mM. Preliminary results from these studies indicate that the pH stability range could be narrowed by increasing H_2O_2 concentration or broadened by decreasing it.

The presence of phenol in the reaction mixture provided the enzyme with some protection against inactivation due to storage at pH 7.0. This conclusion was derived from the studies on CPO stability under storage (this thesis), and the report by Pickard and Hashimoto (1988) on long term storage stability studies of this enzyme at room temperature.

The objectives of this thesis project were reasonably achieved. Chloroperoxidase was covalently immobilized on derivatized glass particles, and this immobilized enzyme preparation showed greater stability under well defined storage and operational conditions. The development of CPO colorimetric assay, based on the H_2O_2-dependent TMPD oxidation, allowed the quantitation of the immobilized enzyme activity. However, some concern still remains regarding the low specific activity of the immobilized enzyme. Whether the reduced activity was an inherent property of the immobilized enzyme or was due to some interference to accurate measurements of bound activity, remains unanswered. More studies are needed to resolve this issue.

The characterization studies of both the immobilized and soluble enzymes not only confirmed some existing properties but also revealed some new properties of CPO. Although the inactivating effect of hydrogen peroxide is well documented (Neidleman 1989), this thesis reports on the dependence of the inactivating effect on the pH, at low concentration of this substrate. The information on the stability of the enzyme, and the distinct separation of the two main activities of CPO: the halide-dependent and halide-independent activities of CPO may have some economic implications. Maloney *et al.* (1986) reported that the halogenase activity of haloperoxidases may limit phenolic oxidation and result in the formation of highly toxic halogenated aromatics. Therefore, it is important to separate the peroxidase and halogenase activities of CPO, if this enzyme is to be used in the treatment of wastewaters. Results presented in this thesis provide encouraging information regarding this problem, since the halogenation activity is maximal at acidic pH. The use of CPO at pH 5.5 to 6.5, where the enzyme is more stable, would favor peroxidation over halogenation. The data from kinetic studies indicated that CPO conforms to the postulated "modified ping-pong" kinetic mechanism (Dunford 1989), and no Michaelis-Menten kinetic constants can be determined. The experiments on H_2O_2-dependent phenol oxidation by soluble CPO were designed to define the optimum operational conditions for efficient use of the immobilized enzyme. Ironically, these studies yielded operational stability data that were more promising for the application of the soluble enzyme, rather than the immobilized enzyme, in phenol oxidation systems. More studies are required to assess the efficiency of phenol oxidation by immobilized CPO in packed-column reactor, rather than in the shaking-batch reactor.

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APPENDIX.

The following equations were kindly provided by Dr. Dunford, after a discussion on CPO kinetics. In the original form, the following were used $(P)_0$, (P), (I), and (II) in equations (1, 2, and 3). Where P stands for peroxidase. And these terms have been substituted with $(CPO)_0$, CPO, CPO-I, CPO-II, respectively. Other minor modifications would appear, but these are limited to replacement of certain terms with others, without compromising the accurate reproduction of the original equations.

$$CPO + H_2O_2 \xrightarrow{k_1} CPO\text{-}I + H_2O \qquad (1)$$

$$CPO\text{-}I + AH_2 \xrightarrow{k_2} CPO\text{-}II + {}^{\bullet}AH \qquad (2)$$

$$CPO\text{-}II + AH_2 \xrightarrow{k_3} CPO + {}^{\bullet}AH + H_2O \qquad (3)$$

where CPO, CPO-I and CPO-II are native enzyme, compounds I and II, respectively; AH_2 is the reducing substrate, and $^{\bullet}AH$ the free radical product. k_1, k_2,and k_3 are rate constants of reactions (1), (2), and (3).

Rate :
$$-\frac{d(AH_2)}{dt} = k_2(CPO\text{-}I)(AH_2) + k_3(CPO\text{-}II)(AH_2) \qquad (4)$$

Conservation:
$$(CPO)_0 = (CPO) + (CPO\text{-}I) + (CPO\text{-}II) \qquad (5)$$

Single steps:
$$-\frac{d(CPO)}{dt} = 0 = k_1(CPO)(H_2O_2) - k_3(CPO\text{-}II)(AH_2) \qquad (6)$$

$$+\frac{d(CPO\text{-}I)}{dt} = 0 = k_1(CPO)(H_2O_2) - k_2(CPO\text{-}I)(AH_2) \qquad (7)$$

$$+ \frac{d(\text{CPO-II})}{dt} = 0 = k_2(\text{CPO-I})(AH_2) - k_3(\text{CPO-II})(AH_2) \qquad (8)$$

From (8): $k_2\,(\text{CPO-I})\,(AH_2) = k_3(\text{CPO-II})\,(AH_2)$

$$k_2\,(\text{CPO-I}) = k_3(\text{CPO-II})$$

$$* \ (\text{CPO-I}) = \frac{k_3}{k_2}\ (\text{CPO-II}) \quad \text{or } (\text{CPO-II}) = \frac{k_2}{k_3}\ (\text{CPO-I}) \qquad (9)$$

From (7): $k_1(\text{CPO})\,(H_2O_2) = k_2(\text{CPO-I})(AH_2)$

$$* \ (\text{CPO}) = \frac{k_2(AH_2)}{k_1\,(H_2O_2)}\ (\text{CPO-I}) \quad \text{or } (\text{CPO-I}) = \frac{k_1\,(H_2O_2)}{k_2(AH_2)}(\text{CPO}) \qquad (10)$$

From (6): $k_1(\text{CPO})\,(H_2O_2) = k_3(\text{CPO-II})(AH_2)$

$$* \ (\text{CPO}) = \frac{k_3\,(AH_2)}{k_1(H_2O_2)}\ (\text{CPO-II}) \text{ or } (\text{CPO-II}) = \frac{k_1(H_2O_2)}{k_3(AH_2)}\ (\text{CPO}) \qquad (11)$$

From (5, 10, and 11):

$$(\text{CPO})_0 = (\text{CPO}) + \frac{k_1(H_2O_2)}{k_2(AH_2)}\ (\text{CPO}) + \frac{k_1(H_2O_2)}{k_3\,(AH_2)}\ (\text{CPO})$$

$$(\text{CPO}) = \frac{(\text{CPO})_0}{1 + \dfrac{k_1(H_2O_2)}{k_2(AH_2)} + \dfrac{k_1(H_2O_2)}{k_3(AH_2)}} \qquad (12)$$

From (5, 9, and 10):

$$(CPO)_0 = \frac{k_2(AH_2)}{k_1(H_2O_2)}(CPO\text{-}I) + (CPO\text{-}I) + \frac{k_2}{k_3}(CPO\text{-}I)$$

$$(CPO\text{-}I) = \frac{(CPO)_0}{1 + \frac{k_2(AH_2)}{k_1(H_2O_2)} + \frac{k_1}{k_3}} \qquad (13)$$

From (5, 9, and 11):

$$(CPO)_0 = \frac{k_3(AH_2)}{k_1(H_2O_2)}(CPO\text{-}II) + \frac{k_2}{k_3}(CPO\text{-}II) + (CPO\text{-}II)$$

$$(CPO\text{-}II) = \frac{(CPO)_0}{1 + \frac{k_3}{k_2} + \frac{k_3(AH_2)}{k_1(H_2O_2)}} \qquad (14)$$

Rate :

$$-\frac{d(AH_2)}{dt} = k_2(CPO\text{-}I)(AH_2) + k_3(CPO\text{-}II)(AH_2) \qquad (4)$$

$$-\frac{d(AH_2)}{dt} = \frac{k_2(AH_2)(CPO)_0}{1 + \frac{k_2(AH_2)}{k_1(H_2O_2)} + \frac{k_2}{k_3}} + \frac{k_3(AH_2)(CPO)_0}{1 + \frac{k_3}{k_2} + \frac{k_3(AH_2)}{k_1(H_2O_2)}}$$

Multiplying by $k_1k_3/(AH_2)$:

$$-\frac{d(AH_2)}{dt} = \frac{k_1k_2k_3(AH_2)\,(CPO)_0}{\frac{k_1k_3}{(AH_2)} + \frac{k_1k_2k_3(AH_2)}{k_1(H_2O_2)} + \frac{k_1k_2k_3}{k_3(AH_2)}} + \frac{k_1k_2k_3(AH_2)(CPO)_0}{\frac{k_1k_2}{(AH_2)} + \frac{k_1k_2k_3}{k_2(AH_2)} + \frac{k_1k_2k_3(AH_2)}{k_1(H_2O_2)}}$$

$$-\frac{d(AH_2)}{dt} = \frac{k_1k_2k_3(CPO)_0}{\frac{k_1(k_2+k_3)}{(AH_2)} + \frac{k_2k_3}{(H_2O_2)}} + \frac{k_1k_2k_3(CPO)_0}{\frac{k_1(k_2+k_3)}{(AH_2)} + \frac{k_2k_3}{(H_2O_2)}}$$

$$V = -\frac{d(AH_2)}{dt} = \frac{2k_1k_2k_3(CPO)_0}{\frac{k_1(k_2+k_3)}{(AH_2)} + \frac{k_2k_3}{(H_2O_2)}} \qquad (15)$$

Where V is the catalytic velocity.

Dividing by $k_1k_2k_3$:

$$\text{or } V = \frac{2(CPO)_0}{\frac{(k_2+k_3)}{k_2k_3}\frac{1}{(AH_2)} + \frac{1}{k_1(H_2O_2)}}$$

Assuming $k_2 > k_3$

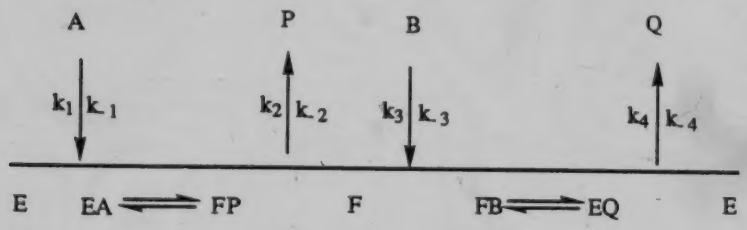
$$V = \frac{2(CPO)_0}{\frac{1}{k_3}\cdot\frac{1}{(AH_2)} + \frac{1}{k_1(H_2O_2)}}$$

$$\frac{V}{2(CPO)_0} = \frac{1}{\frac{1}{k_3}\cdot\frac{1}{(AH_2)} + \frac{1}{k_1(H_2O_2)}}$$

$$\frac{2(CPO)_0}{V} = \frac{1}{k_3(AH_2)} + \frac{1}{k_1(H_2O_2)} \qquad (16)$$

A. In normal ' Ping - Pong ':

Individual steps using Cleland notation (Segel 1975)



Where A and B are the two substrates; P and Q the products; and E and F the two enzyme species. k_1 to k_{-4} are the rate constants of forward and backward reaction steps.

Initial forward velocity in the absence of products:

$$\frac{V}{(E)_0} = \frac{k_{cat}(A)(B)}{Km_{(A)}(B) + Km_{(B)}(A) + (A)(B)} = \frac{k_{cat}}{1 + \frac{Km_{(A)}}{(A)} + \frac{Km_{(B)}}{(B)}}$$

$$\frac{(E)_0}{V} = \frac{Km_{(A)}}{k_{cat}} \frac{1}{(A)} + \frac{1}{k_{cat}} \left(1 + \frac{Km_{(B)}}{(B)}\right)$$

Where $Km_{(B)}$ and $Km_{(A)}$ are Michaelis constants for substrates B and A , respectively. k_{cat} is the maximum theoretical rate constant.

By holding B constant, and varying (A); then choosing new B to hold constant, and varying (A) again, and so on, parallel lines would be obtained in the original plots of $(E)_0/V$ vs 1/B. From the replots of intercepts from original plots and 1/B, k_{cat} and $Km_{(B)}$ could be derived form the intercept with the y-axis and the slope, respectively.

The ordinate intercepts of the parallel lines are given the following equation:

$$\text{intercept} = \frac{Km_{(B)}}{k_{cat}} \frac{1}{(B)} + \frac{1}{k_{cat}}$$

The slope equation of the replots:

$$\text{Slope} = \frac{Km(B)}{k_{cat}}$$

B. In normal 'Peroxidase Ping - Pong ':
(from 16)

$$\frac{2(E)_0}{V} = \frac{1}{k_3 (AH_2)} + \frac{1}{k_1(H_2O_2)}$$

First by holding H_2O_2 constant, and varying AH_2, then choosing new H_2O_2 to hold constant, and varying AH_2; parallel lines would be obtained in the original plots of $(E)_0/V$ vs $1/AH_2$. From the replots of intercepts from the original plots and $1/H_2O_2$, k_{cat} and $Km(H_2O_2)$ could not be derived since this secondary plot has a zero intercept. The ordinate intercepts of the parallel lines are given the following equation:

$$\text{intercept} = \frac{1}{k_1(H_2O_2)}$$

The slope equation of the replots:

$$\text{Slope} = \frac{1}{k_1}$$

Although Km values cannot be derived from the plots for CPO since the peroxidase cycle is irreversible, the rate constants k_1 and k_3 can be determined from steady state kinetics.